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Title of Thesis: Ethanol-drug Metabolic Interactions

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ABSTRACT

Title of Dissertation: Ethanol-Drug Metabolic Interactions

Robert G. Elves, Doctor of Philosophy, 1984

Dissertation directed by: Alvito P. Alvares, Professor, Department of Pharmacology

The present studies examined the effects of acute and chronic ethanol administration on the N-acetylation of arylamine containing drugs in two strains of mice which have been shown to be "rapid" and "slow" acetylators. In humans, differential toxicity of some drugs which undergo acetylation is dependent upon acetylator phenotype. Genetic slow acetylators are more prone to develop drug-induced systemic lupus erythematosis and polyneuropathy. Rapid acetylators are more prone to liver damage. The administration of ethanol to humans has been shown to increase the acetylation rate of sulfamethazine (SMZ) and procainamide (PA). Ethanol is metabolized to acetate, and theroretically could increase AcCoA levels, the acetate donor for the N-acetyltranferase (NAT) reaction. This could lead to increased acetylating capability by the liver and other tissues. To aid in the study of the human acetylation-ethanol interaction, the mouse models A/J and C57BL/6J (C57) strains, which have previously been shown to exhibit acetylation polymorphism, were studied utilizing a newly developed ethanol-containing liquid diet. Prior to determining the regulation of acetylation by ethanol, the mice were first characterized for the inductive effects of the classical microsomal enzyme inducers (PB), 3-methylcholanthrene (3-MC), and the polychlorinated biphenyl mixture, Aroclor 1254 (PCBs) to establish a basis of comparison with the ethanol induction studies. Following the characterization of

the hepatic monooxygenases, a comparison was made of the inductive properties of ethanol in these mouse models. Finally the effects of acute and chronic ethanol administration on the acetylation rates of several test drugs were determined in the mouse model.

The inducing properties of chronic ethanol ingestion on hepatic monooxygenases in Sprague-Dawley and Long-Evans rats, and A/J and C57 mice, were studied. Cytochrome P-450 contents were increased in livers in all animals receiving the experimental ethanol-containing liquid diet. The CO-difference spectra of microsomes from ethanol-treated animals showed a shift in the absorbance maximum of 450 nm observed with microsomes from control animals. Ethylmorphine N-demethylase and benzo[a]pyrene hydroxylase activities in livers of ethanol-treated animals were minimally affected. The shift in the absorbance maxima to longer wavelengths in the CO-difference spectrum and the minimal effects on the metabolism of ethylmorphine and benzo[a]pyrene, demonstrate that ethanol differs in its inducing properties, when compared to the properties of the two classical hepatic microsomal enzyme inducers PB and 3-MC. PB and PCBs both increased cytochrome P-450 content and increased N-demethylase activity and caused minimal changes in benzo[a]pyrene activity in livers of both strains. 3-MC increased benzo[a]pyrene activity in both strains, and caused a CO-difference spectral shift to 448 nm only in the C57 strain. In contrast to the minimal effects observed on the metabolism of ethylmorphine and benzo[a]pyrene, ethanol pretreatment increased hepatic 7-ethoxycoumarin O-deethylase and aniline hydroxylase activities in the treated animals. Polyacrylamide gel electrophoresis of hepatic microsomes from those animals receiving ethanol revealed protein band(s) in the cytochrome P-450 molecular weight region, the intensities of

which were markedly increased relative to that from control animals. The heme-associated peroxidase activity was also increased in the same molecular weight region. Induction by another aliphatic alcohol, isopropanol, in the Sprague-Dawley rat was similar to the induction produced by ethanol. The results of the present spectral, catalytic, and electrophoretic studies demonstrate that in mice, as in rats, chronic ethanol treatment causes the induction of specific cytochrome(s) P-450 with preferential activity toward aniline and 7-ethoxycoumarin.

The erythrocyte NAT studies showed that SMZ was a poor substrate for this enzyme, whereas p-aminobenzoic acid (PABA) showed high activity and was polymorphically acetylated. Neither acute nor chronic ethanol pretreatment altered the acetylation of PABA in the two mouse strains. Therefore, there was no apparent induction of NAT activity following ethanol pretreatment in the mice. Both acute and chronic ethanol treatments increased the urinary excretion ratio of acetylated to parent compound for SMZ in the A/J mice, and sulfanilamide (SNL) and PA in the C57 mice. Acute ethanol pretreatment increased the elimination rate of SMZ but not SNL. The present studies indicate that A/J and C57 mice, under certain conditions, may model the increases in acetylation rate seen during ethanol consumption in man. However, the mouse model showed substrate and strain dependence which was independent of phenotype or previous polymorphism. The increases in acetylation noted in man or animals is most likely due to the subsequent metabolism of ethanol to AcCoA, the rate limiting factor for NAT. Since serious drug toxicities are associated with rapid and slow acetylator phenotypes and ethanol may affect acetylation rate, ethanol consumption is a clinically significant parameter which must be considered prior to exprosure to drugs or other compounds metabolized by acetylation.

ETHANOL-DRUG METABOLIC INTERACTIONS

by

Robert G. Elves

Disertation submitted to the Faculty of the Department of Pharmacology
Graduate Program of the Uniformed Services University of the
Health Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1984

DEDICATION

To my Father and Grandmother, who have showed me how to endure adversity.

"Never take a simple 'no' for an answer"

To my wife Teresa and my Mother, two women whose support quelled the lows and sustained the highs. To my two daughters, Sheila-Ann and Kristin, who some day might understand why their Dad couldn't always be home.

ACKNOWLEDGEMENT

I wish to thank the United States Air Force, the Air Force Institute of Technology, and F. Edward Hébert School of Medicine for providing the research opportunity and financial support. To Dr. Alvito P. Alvares, my mentor, who provided research support and guidance. To the Department of Pharmacology, who provided the facilities and helpful advise.

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ABBREVIATIONS

The following abbreviations will be used throughout the text:

AcCoA	-	acetyl coenzyme A
AcSMZ	-	acetylsufamethazine
AcSNL	-	acetylsulanilamide
C57	-	C57 BL/6J mouse
HPLC	-	high performance liquid chromatography
3-MC	-	3-methylcholanthrene
NAPA	-	N-acetylprocainamide
NAT	-	N-acetyltransferase
PA	-	procainamide
PB	-	phenobarbital
PCBs	-	polychlorinated biphenyls
SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDZ	-	sulfadiazine
SMZ	-	sulfamethazine
SNL	-	sulfanilamide

INTRODUCTION

A. Acetylation Polymorphism and Toxicology: The rate of metabolism of certain drugs, primarily arylamines, by an acetylation pathway has shown a genetically controlled polymorphism, both in animals and humans (see review by Lunde et al., ref.1). This metabolic pathway is controlled by an enzyme, N-acetyltransferase (NAT), which acetylates a number of arylamine compounds. Large inter-ethnic variations in the frequency of rapid and slow acetylators have been found (Table 1). Generally Caucasian populations are comprised of approximately 50% slow acetylators, while ethnic groups of Oriental origin are mainly rapid acetylators (Table 1).

However, not all drugs which are acetylated demonstrate genetically determined rates of metabolism. Some drugs are acetylated polymorphically while others show only monomorphic rates of acetylation. In man, drugs such as isoniazid, hydralazine, procainamide (PA), and sulfamethazine (SMZ) are metabolized polymorphically, whereas drugs such as sulfanilamide (SNL) and p-aminobenzoic acid (PABA) are metabolized monomorphically (8).

The two acetylator phenotypes are associated with differential human drug toxicities. Slow acetylators accumulate drugs if the agents are administered under prolonged dosage regimens. Peripheral neuropathy is the sequelae common with isoniazid treatment of slow acetylators, and is believed to be caused by biological accumulation of the drug (79). Slow acetylators of certain drugs, such as isoniazid (80), hydralazine (81,82), and PA (2) are predisposed to drug-induced lupus erythematosus and associated findings of antinuclear antibodies. An antinuclear antibody

Table 1. Incidence of slow acetylators in some ethnic groups and populations[a]

Ethnic Group	Slow Acetylators[b] (%)
Egyptians	82
Swedish	68
Finns	61
Canadian White	59
U.S. White	53
Skolt Lapps	50
Canadian Indian	44
Africans	41
U.S. Black	40
Philippines	28
Chinese	22
Saame Lapps	20
Japanese	12
Eskimos	0

[a] Data taken from Olsen (151).

[b] Individuals phenotyped with sulfamethazine.

assay has been used as a diagnostic test of systemic lupus erythematosis. Recent studies (3,4) indicate that there may be a correlation between slow acetylators and idiopathic lupus erythematosis and it has been speculated that such individuals may inadvertantly be exposed to environmentally-derived xenobiotics which undergo acetylation reactions similar to PA and isoniazid (8).

Fast acetylators may require larger doses of drugs to achieve the desired therapeutic effect if the metabolites of the drug are inactive; whereas the drug dose may need to be decreased when such drugs produce pharmacologically active acetylated metabolites. In addition to a compound having pharmacologically active metabolites the drug may also have toxicologically active metabolites. A significant proportion of rapid acetylators receiving long-term isoniazid therapy develop an isoniazid-associated hepatitis and general liver toxicity (5). Isoniazid is partly metabolized to monoacetylhydrazine which has been postulated to cause liver necrosis in humans and experimental animals (83). 25-28% of isoniazid is excreted as the acetylated metabolite in rapid acetylators and 5-8% in slow acetylators (84).

Because of the toxicities associated with certain drugs that undergo acetylation, there is a need to characterize a patient's phenotype, and in some cases to monitor the patient's drug level to obtain the desired therapeutic effect. Because of the multiple metabolic pathways found with most of the drugs involved, humans are phenotyped using SMZ (6,7), or dapsone (7).

B. Animal Models in Biomedical Research: Models are an essential part of biomedical research and have been used since the first experiments were performed. Baldissarini and Fischer (114) suggested that models are

an experimental compromise, a simple experimental system used to represent a much more complex and less readily available system. It is usually preferable, if not mandatory, to employ animal models rather than use human subjects. Much research cannot be performed in man for ethical reasons. For metabolic studies, animals provide a large number of identical individuals with little intra-individual variation, unlike the human population. The genetic background may also be controlled in animals in contrast to the human situation. In addition, drug metabolism in man is affected by a number of environmental factors which an investigator may not be able to control (115). In animal studies environmental factors can either be controlled or standardized. The next sections describe the use of several animal models used to simulate the human disease condition.

1. Animal Models of Human Acetylation Polymorphism: In the past several years a number of species have been investigated as models of human acetylator polymorphism (see review by Weber and Glowinski, ref. 8). Studies to date demonstrate that acetylation polymorphism is dependent on species, substrate, tissue, and if the procedure was performed in vivo or in vitro (Table 2). Acetylation polymorphism for the metabolism of several compounds has been found in man, monkey, and rabbit. The rat and baboon show essentially no acetylation polymorphism for the metabolism of these compounds. The inbred hamster is rather unique in that it has been shown to have acetylator polymorphism for PABA, but not for isoniazid, SMZ or PA (9). These effects are in direct contrast to the those found in man or rabbits.

Commonly the rabbit has been used to study isoniazid-acetylator polymorphism. The disadvantages in using the rabbit model are numerous. The New Zealand White rabbit, an outbred animal, is used for this purpose.

Table 2: Substrate and tissue dependency of N-acetylation in mammalian species[a]

Acetylation parameter	Substrate	Species[b] Man	Rabbit	Rat	Mouse
Urinary excretion of AcSMZ	SMZ	B	B	ND	B
Liver NAT activity	SMZ	B	B	ND	U
	PABA	U	U	U	U
Erythrocyte NAT activity	PABA	U	B	B	B

[a] Data taken from Glowinski and Weber (8)

[b] Parameter measured in each species was found to be bimodal (B), unimodal (U), or was not determined (ND). Within the given species if the individual animals can be separated into two subsets of acetylator phenotypes then acetylation polymorphism exists and species is designated bimodal.

However, each animal needs to be phenotyped using a test drug and a large number of each phenotype must then be kept on hand. Monkeys and deer mice must also be individually phenotyped. The purchase and maintainance of the large colonies of these species required for experimental study are expensive.

2. Mouse Model of Human Acetylator Polymorphism: The mouse strains, C57BL/6J (C57) and A/J from Jackson Laboratories have been shown to be a useful genetic model of PA-induced systemic lupus erythematosis (10). The C57 strain was classified as rapid acetylators and the A/J strain as slow acetylators based on how readily they acquired the disease. In characterizing these two strains, polymorphism in the N-acetylation of arylamine drugs and carcinogens was also found (11). The most important finding with these strains was the acetylator polymorphism noted with several substrates (Table 3). A/J mice have low erythrocyte PABA NAT activity and C57 mice have a high erythrocyte PABA NAT activity. A/J mice excrete urine which contains a low ratio of AcSMZ to SMZ in urine and C57 mice excrete urine with a higher ratio of AcSMZ to SMZ urine (12,13). It has been shown that PA is acetylated to a lesser degree in A/J than in C57 mice as measured in erythrocyte preparations. However, no significant differences in urinary excretion of acetylated PA were noted in these two strains (10).

In some respects the acetylator characteristics of the mice are different from man. For example man shows no polymorphism for PABA or for any other substrates when erythrocyte preparations are used. Rabbits show polymorphic characteristics very similar to man. However, in contrast to man, but similar to mice, rabbits show polymorphism of PABA. Thus, there is as yet no single animal model which mimics the

Table 3: A/J and C57 mouse acetylation characteristics

Substrate	N-acetyltransferase activity[b]		Urinary Excretion of acetylated metabolite[c]
	Erythrocyte	Liver	
PABA	C57>A/J	C57=A/J	C57=A/J
SMZ	low activity	C57=A/J	C57>A/J
PA	C57>A/J	not determined	C57=A/J

[a] Data taken from Glowinski and Weber (8) and Tannen and Weber (11).

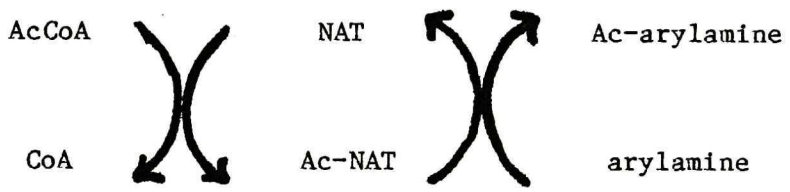
[b] *In vitro* determination of acetylation activity.

[c] *In vivo* determination of acetylation activity dependent on amount of acetylated compound excreted.

NOTE: Bimodal activity exists when C57>A/J and unimodal activity when C57=A/J.

human polymorphism in all respects. Although the mouse model does not entirely mimic the human situation, it allows the study of acetylation characteristics of several substrates and the regulation of the N-acetylation reaction. Mice can also be purchased at a relatively low cost compared with the cost of rabbits and are readily procured.

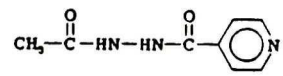
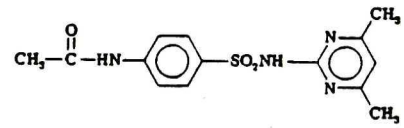
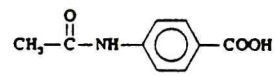
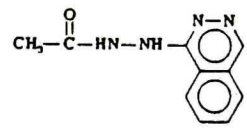
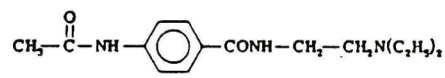
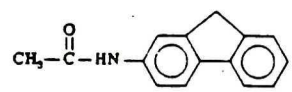
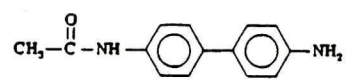
C. N-Acetyltransferase: Most studies involving NAT use rabbit liver as the source for this enzyme (8). NAT, a cytosolic enzyme, has the highest activity in liver. NAT is also found in the small intestine, kidney, and erythrocyte and these extrahepatic sources of the enzyme may significantly contribute to the overall metabolism of a compound in the intact animal (14). Activity is not detected in plasma, fat, or skeletal muscle. Drugs that are acetylated by NAT are shown in Table 4. This enzyme catalyzes the acetylation of the $-NH_2$ group.



Two sequential steps were required for the process. The first is the donation of an acetyl group from AcCoA to the enzyme to form an acetyl-enzyme intermediate; in the second step the intermediate gives up the acetyl group to the substrate with regeneration of the enzyme and production of the acetylated substrate. The formation of the intermediate appears to be the rate determining step in the acetylation process (20,21). Therefore, V_{max} of the reaction occurs when both substrates are at infinite concentrations.

Rabbit liver homogenates have been used to determine the kinetic properties of the enzyme (19,20). It was found that AcCoA was the acetyl

Table 4: Some drugs and other compounds which are biotransformed by acetylation[a]

Compound	Acetyl derivative
Isoniazid	
Sulfamethazine	
p-Aminobenzoic Acid	
Hydralazine	
Procainamide	
Aminofluorene	
Benzidine	

[a] Data taken from Glowinski and Weber (8).

donor for a number of arylamine compounds. NAT activity was inactivated by sulfhydryl reagents and inhibited by the reaction product coenzyme A (8). Competitive inhibition of NAT was found with other arylamines and by amethopterin (15,16). Based on initial velocities, product inhibition and isotope exchange studies, acetylation occurs by a "ping-pong" Bi-Bi reaction mechanism.

NAT has been purified from the 100,000 x g supernatant of liver homogenates prepared from the livers of rabbits and mice (19,74). Purified rabbit liver NAT retains 80% of its original activity at pH 7.2 stored at 4° for 4 to 5 days (116). Otherwise preparations withstand freezing without appreciable losses. Rabbit liver NAT has a molecular weight of 37,000 (17) and the molecular weight of human NAT has been estimated at 26,500 (18).

Recent findings indicate that the monomorphic and polymorphic NAT activites coexist on a common protein (131). The two activities could not be separated by two extensive purification procedures involving sequential centrifugation, fractional precipitation, ion-exchange chromatography, gel filtration, isoelectric focusing, or by polyacrylamide electrophoresis. In addition, heat activation patterns were identical.

D. Acetylation Polymorphism and Ethanol Interaction: Acetylation polymorphism is important in drug therapy because of the serious drug toxicities associated with both the rapid and slow phenotypes. Any factor or condition which would affect acetylation rate would thus be of clinical significance. In 1964, Lester (49) reported that chronic alcohol (ethanol) abuse may affect the rates of acetylation in man. His study included a group of chronic alcohol abusers who were phenotyped with isoniazid. Although these individuals could be separated into two

distinct groups based on isoniazid half-life, both groups showed a reduced half-life for isoniazid when compared to the normal population. Next, two normal controls, one a rapid acetylator and one a slow acetylator were administered isoniazid while consuming high oral doses of ethanol. Both individuals showed a reduced half-life for isoniazid. These data provided the first evidence that ethanol ingestion may alter the elimination rate of a drug that undergoes acetylation in the body.

Studies investigating the ethanol-acetylation interaction have produced conflicting data. A study in rats showed that ethanol retards the rate of absorption of isoniazid from the gastrointestinal tract, but increased N-acetylisoniazid excretion (120). Other studies have shown that ethanol administered intravenously enhanced the amount of N-acetylisoniazid in the urine, but did not affect the half-life of isoniazid (121). However, studies in guinea pig (122) and rats (123), did not show any effects of ethanol on isoniazid or SMZ acetylation.

Recently two studies carried out in humans investigated effects of acute ethanol on the acetylation of SMZ (50) and PA (51). Ethanol increased the percentage of SMZ acetylated and also decreased the half-life of SMZ (Table 5). SMZ half-life was decreased 23% in rapid acetylators and 17% in slow acetylators. In another study (51), 7 slow and 11 rapid acetylators respectively were phenotyped, using PA as the test substance (see Table I, Olsen & Morland, ref. 51). The investigators subsequently selected 5 slow and 6 rapid rapid acetylators, respectively, for an acute ethanol/ PA study. Acute ethanol administration caused a significant reduction in half-life and a significant increase in total clearance of PA. These changes may be due to increased metabolism (enhanced

Table 5: Effect of acute ethanol on sulfamethazine pharmacokinetics[a]

	Control	Ethanol
Apparent half-life (min)		
Slow acetylators	301 ± 20	250[b] ± 22
Rapid acetylators	129[c] ± 4	99[b] ± 3
Percentage of SMZ acetylated in 8 hr[c]		
Slow acetylators	30.0 ± 1.1	36.7[b] ± 2.9
Rapid acetylators	79.3[c] ± 1.9	84.7[b] ± 1.9

[a] Individuals were given 10 mg/kg of the drug. Two hours later half the subjects received no further treatment, while the others had ethanol (20% v/v in fruit juice, 0.73 g/kg) followed by hourly drinks of 0.11 g/kg until 10 hours after the drug administration. Each value represents the mean ± S.E. for at least 5 subjects.

[b] Value significantly different from the respective control value ($p < 0.05$)

[c] Value significantly different from respective A/J value ($p < 0.05$)

Data taken from Olsen and Morland (50)

Table 6: The effect of acute ethanol on procainamide pharmacokinetics[a]

	Control	Ethanol
Apparent half-life (min)		
Slow acetylators	186 ± 38	138[b] ± 28
Rapid acetylators	153 ± 43	120[b] ± 21
Total clearance (ml/min)		
Slow acetylators	472 ± 108	719[b] ± 169
Rapid acetylators	582 ± 138	704[b] ± 169

[a] In data not shown in this Table, 7 slow and 11 rapid acetylators were phenotyped using PA as the test substance (see Table I, Olsen & Morland, ref. 51). The investigators subsequently selected 5 slow and 6 rapid acetylators and treated as follows: individuals were given 10 mg/kg of the drug. Two hours later half the subjects received no further treatment, while the others had ethanol (20% v/v in fruit juice, 0.73 g/kg) followed by hourly drinks of 0.11 g/kg until 10 hours after the drug administration. Each value represents the mean ± standard deviation for at least 5 subjects.

[b] Value significantly different from the respective control value ($p < 0.05$). Data were analyzed by Wilcoxon's test for paired samples.

enzyme activity, increased cofactor, or drug concentrations at the site), and/or changes in excretion. It could be speculated that the increased acetylation caused by ethanol was due to increased formation of AcCoA from acetate during ethanol metabolism. AcCoA has been shown to be the required cofactor for NAT which catalyzes the acetylation of the above studied drugs. In vitro studies have shown that acetylation rate can be increased by increasing the concentration of AcCoA (64). In vivo studies have shown that ethanol may increase the cellular concentration of AcCoA (124,125). Since serious drug toxicities are associated with rapid and slow acetylator phenotypes and ethanol may affect acetylation rate, ethanol consumption is a clinically significant parameter which must be considered prior to exprosure to drugs or other compounds metabolized by acetylation.

The exact mechanism of ethanol's effect on acetylation reactions is at present unclear and open to further research. Research in this area would benefit from the development of an animal model of the human acetylation polymorphism-ethanol interaction. An animal model would allow separation of the variables and provide a means of investigating the regulatory mechanisms.

E. Pharmacology of Ethanol: Since the experimental problem described involves the administration of ethanol either acutely or chronically, it is appropriate to review ethanol pharmacology. Research in this area is substantial considering that ethanol is a simple two carbon alcohol. The pharmacology of ethanol has been generally reviewed by Goldstein (85) and extensively reviewed by Lieber (86). Therefore, only those studies most pertinent to the present objective will be briefly reviewed.

The pharmacological and biochemical properties of ethanol are related to the rate of metabolism of ethanol to acetate via a toxic intermediate, acetaldehyde (22). Hence, an understanding of the regulation of ethanol oxidation is essential to the study of ethanol interactions. Ethanol administered orally is primarily absorbed from the gastrointestinal tract by diffusion. After absorption, ethanol is distributed in total body water. The rate of distribution within the brain, liver, lungs and kidney is rapid due to high blood flows in these organs. It should be noted that in the absence of ethanol ingestion, trace amounts of ethanol have been found in the body, derived from bacterial fermentation in the gut and possible synthesis by endogenous metabolic routes (23,39). Endogenous ethanol is metabolized by the hepatic microsomal ethanol oxidizing system, and other ethanol oxidizing metabolic pathways in untreated animals. In humans, at low concentrations of ethanol, less than 2% is excreted in expired air, urine, and sweat. As levels rise above 200 mg/dl renal and pulmonary excretions may total 15%. Other human studies have shown that 90% of ethanol elimination is through liver metabolism (24,25). Ethanol metabolism for a 70 kg man can vary from 200 to 240 g/day, and for the chronic alcoholic it can be as high as 370 g/day (25).

1. Ethanol Metabolism: Ethanol metabolism was thought to occur almost exclusively in the liver hepatocyte and only small amounts of ethanol were metabolized outside the liver (113). Recently, incubated rat lung slices were shown to metabolize ethanol (126). The authors speculated that lung may have the second highest activity to metabolize ethanol next to liver (126). Because little additional information is available on this phenomena, only information pertinent to liver metabo-

lism will be reviewed.

The hepatocyte contains three enzyme systems that metabolize ethanol to acetaldehyde (Fig. 1): alcohol dehydrogenase in cytosol, catalase in peroxisomes, and the microsomal ethanol oxidizing system in endoplasmic reticulum. Acetaldehyde is then metabolized by a mitochondrial enzyme, aldehyde dehydrogenase, requiring NAD^+ as a cofactor. The final products of acetaldehyde metabolism are NADH and acetate, although acetate is rapidly converted by AcCoA synthase to AcCoA, which in turn is metabolized via the Krebs cycle to CO_2, NADH, and ATP. AcCoA is also available for other metabolic reactions including its role as the acetyl donor for NAT.

a. Alcohol Dehydrogenase: The primary enzymic pathway responsible for most hepatic ethanol oxidation is alcohol dehydrogenase. Alcohol dehydrogenase catalyzes the oxidation of ethanol to acetaldehyde and requires NAD^+ as a cofactor (Fig. 1). In vivo ethanol metabolism generally proceeds at a linear rate once absorption is complete. This was once thought to be due to alcohol dehydrogenase having a low K_m for ethanol (2 mM), and the enzyme was easily saturated (66). Therefore, once the enzyme was saturated, ethanol metabolism followed zero order kinetics.

Recent studies have suggested that alcohol dehydrogenase is not necessarily the rate limiting step in ethanol metabolism (26-28). The cellular availability of NAD^+ is presently being considered to be the rate-limiting factor in ethanol metabolism. NAD^+ must be regenerated from NADH, which occurs by a variety of NAD^+-dependent dehydrogenases, and the rate of this process is determined by the activity of these enzymes and the availability of the oxidized forms of their substrates.

In the normal pathway acetaldehyde is further oxidized via aldehyde

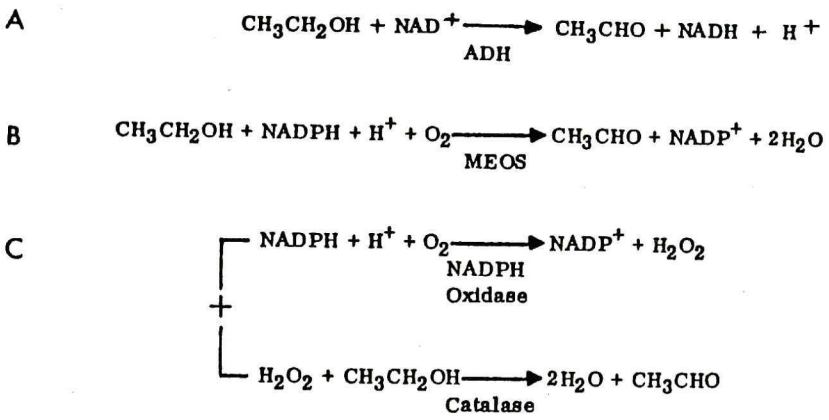


Fig. 1. Ethanol oxidation by A, alcohol dehydrogenase (ADH) and nicotinamide adenine dinuceotide (NAD); B, the hepatic microsomal ethanol oxidizing system (MEOS) and NADPH; C, a combination of NADPH oxidase and catalase.

dehydrogenase to acetate. In the process, NAD^+ is again reduced to NADH. NADH formed is reoxidized to NAD^+ by the respiratory enzymes. Most of the oxygen delivered to the liver during ethanol metabolism is utilized for oxidation of ethanol (87).

Ethanol produces several metabolic effects when the NAD^+/NADH ratio is markedly decreased. Changes in NAD^+ and NADH levels lead to ketosis, acidosis, hyperuricemia, hypoglycemia, and triglyceride synthesis as shown in Fig. 2 (29-34).

b. Catalase: Catalase, found in peroxizomes, metabolizes ethanol to acetaldehyde in the presence of endogenously produced hydrogen peroxide (Fig. 1). This system plays a minor role in ethanol oxidation, even at high dosages of the alcohol. This conclusion was supported by the evidence that ethanol metabolism was unaffected by catalase inhibition (45).

c. Microsomal ethanol oxidizing system: A major advance in the field of alcohol research was the recognition in 1968 of an accessory pathway of ethanol metabolism in the hepatocyte, the microsomal ethanol oxidizing system (35,36). This associated with this system is similar to other mixed function oxidases located in the endoplasmic reticulum. Using reconstitution experiments, it has been shown that the microsomal ethanol oxidizing system requires cytochrome P-450, cytochrome P-450 reductase, molecular oxygen, NADPH and phospholipid for activity (36). The K_m for this reaction has been reported between 8-20 mM ethanol (86). This enzyme becomes the major route of metabolism at higher levels of ethanol when the alcohol dehydrogenase system is saturated.

Both acute and chronic interactions of ethanol with drugs are widely recognized (38). These interactions have been studied primarily

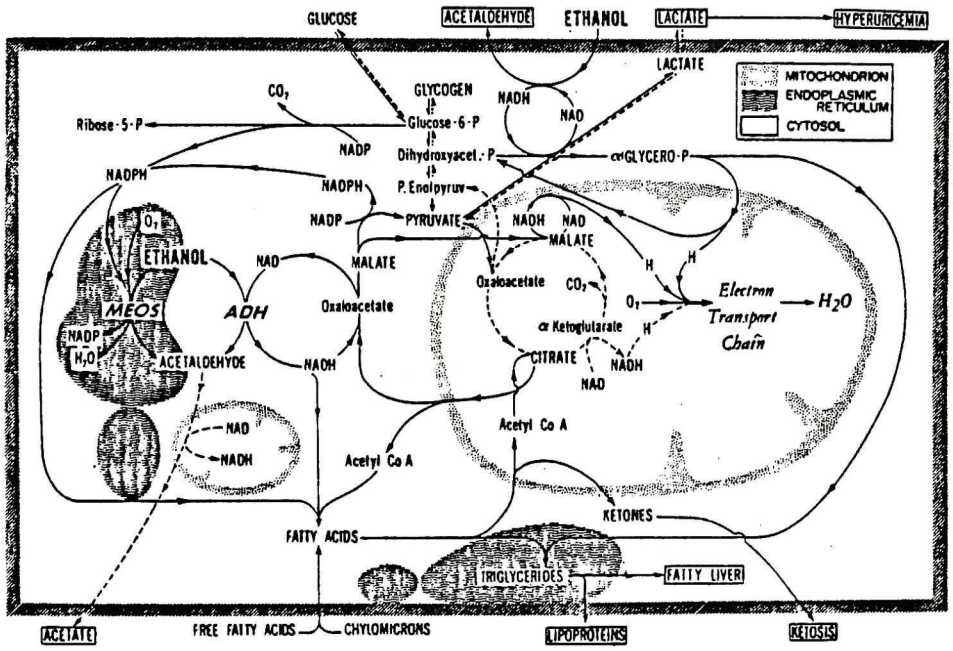


Fig. 2. Metabolism of ethanol in the hepatocyte. Pathways that are decreased after ethanol abuse are represented by the dashed lines. Reproduced from Lieber (86) with permission.

with the oxidative pathways of drugs metabolized by the cytochrome P-450-dependent monooxygenases. The main effect of the acute presence of ethanol is the inhibition of drug oxidations. These drugs are metabolized by hepatic drug metabolizing enzymes localized in the endoplasmic reticulum and appear to be competative enzyme inhibitors of the microsomal ethanol oxidizing system (40). Ethanol indeed inhibits the oxidative metabolism of a variety of drugs in vitro (88-90). In vivo, acute oral administration of ethanol decreases the rate of oxidation of several drugs (88,91).

In contrast to the acute effects of ethanol, chronic ingestion of ethanol enhances the activities of a variety of microsomal mixed function oxidase drug metabolizing enzymes (40-44). Controlled studies in man have clearly shown increases in the rate of metabolism of meprobamate, pentobarbital (43), aminopyrine (44), tolbutamide (42), and propranolol (46). Increased metabolism of xenobiotics can also result in formation of toxic metabolites. This may well be the effect seen in the observed interactions of ethanol with CCl_4 and acetaminophen, to produce hepatotoxicity (47,48). In our own studies (126), isopropanol increases one or more forms of cytochrome P-450 which selectively enhance the metabolism of CCl_4. The metabolite then causes a nonselective damage to the microsomal mixed function oxidase system.

2. Animal Models of Alcohol Dependence: A number of studies have been performed to develop an ideal model to study the pharmacology of ethanol in experimental animals. Criteria for such a model would include voluntary intake of ethanol (perferably by an oral route) such that tolerance, physical dependence, and relapse after abstinence would occur. Most studies used either rats or monkeys as test animals. These studies have been thoroughly reviewed by Myers and Veale (92). In the

rat experiments ethanol was usually administered in the drinking water. Monkeys received ethanol by self-administration usually through behavioral reinforcement techniques.

A difficulty in animal experiments was that the high doses of ethanol required to show any effects usually displaced the normal food calories of the diet. If animals were given ethanol in their drinking water, by injection, or by intubation the treated animals would eat less solid food than the control group. Ideally, in metabolic studies both groups should receive the same caloric intake with the same proportions of fat, protein, and carbohydrate in the diet.

a. Nutritionally Adequate Diet for Rats: In 1967, DeCarli and Lieber (75) developed a diet which contained optimal amounts of fat and protein, to which either ethanol or a carbohydrate could be added in isocaloric amounts. The animals were then pair fed such that both the control and ethanol-treated groups received exactly the same amounts of nutrients. This diet has been successfully used not only in rats but also in primates (93,129).

b. Nutritionally Adequate Diet for Mice: Many attempts have been made in useing the diet and feeding regimen of DeCarli and Lieber in experments using mice, but these experiments have met with mixed success. The initial attempt to treat mice with the ethanol diet, developed for rats, was unsuccessful. The mice were unable to adapt to increases in the ethanol concentration of the original diet. Petersen et al. (52) adapted mice to the DeCarli and Lieber diet by increasing the concentration of ethanol more gradually than previously used for rats.

F. Hepatic Microsomal Drug-Metabolizing Enzyme: Most studies involving drug metabolism have centered around the liver microsomal

hemeproteins collectively called cytochrome P-450. Cytochrome P-450 has been identified as the terminal oxidase of the hepatic microsomal drug-metabolizing system (94,95). It was shown to be the substrate binding site for hydroxylation reactions (96,97). Unlike most enzymes which are highly specific for substrates belonging to one chemical class, cytochrome P-450 acts on a large number of diverse compounds. Another unique feature of cytochrome P-450 was the ability to enhance its activity in animals and man by drugs and various environmental compounds which were accordingly called microsomal enzyme inducing agents. The magnitude of this enhancement was dependent on the inducing agent, dose of agent, species or strain of animal, and the substrates used to monitor the increased activity. Increased microsomal activity can markedly alter the pharmacology and toxicology of various compounds.

1. Induction Studies: Different inducers have been used to manipulate the chemical and physical properties of cytochrome P-450(s). Multiple forms of cytochrome P-450 have been isolated and purified from different tissues of different animal species. These cytochrome P-450s have been shown to differ in their spectral properties, catalytic activity towards different substrates, electrophoretic mobilities, immunological properties, and amino acid sequence (for reviews see 98,103-105). Inductive properties of various compounds can be distinguished by the ability of certain agents to elevate levels of specific cytochrome P-450s (106). These changes could not be produced by simply adding the inducing agents to the rat liver microsomes (109). Additionally, when prototypes of the two major classes of inducing substances (see below) were given together to animals, the effects were additive (110).

Inducers of hepatic monooxygenases have, in general, been categorized into two classical groups. One group of inducers, of which phenobar-

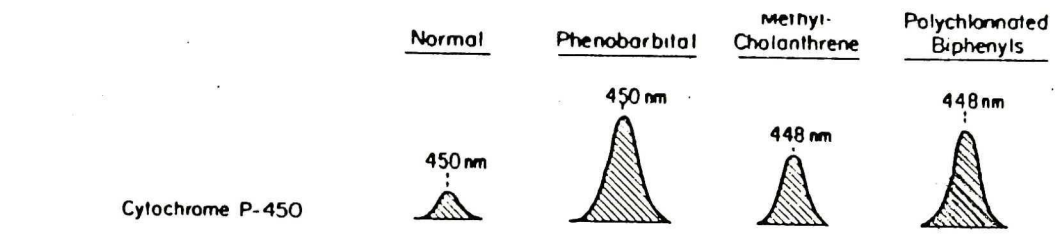
bital (PB) is the prototype, enhances the metabolism of a large variety of substrates by liver cells; a second group consisting of polychlorinated hydrocarbon inducers, of which 3-methylcholanthrene (3-MC) is the prototype, stimulates the metabolism of only a few types of substrates.

A third class of inducers, the polychlorinated biphenyls (PCBs) mixture (Aroclor 1254), possesses the inducing properties of both the PB and 3-MC classes of inducers in rat microsomes (102). The most commonly studied PCB mixture is Aroclor 1254, which has a chlorine content of 54% and contains primarily tetra-, penta-, and hexachlorobiphenyls (101).

A summary of changes in spectral and catalytic properties of liver microsomes from rats pretreated with the various inducers is depicted in Fig. 3, with additional spectral, catalytic, and electrophoretic properties presented in the following paragraphs.

a. Effects of Inducers on Spectral Properties: All three inducers increase the cytochrome P-450 levels; however the reduced carbon monoxide difference spectra of microsomes show absorption maxima at 450, 448, and 448 nm following treatment with PB, 3-MC, and Aroclor 1254, respectively. Cytochrome P-450 from untreated rats showed spectral properties similar to those of the PB induced form (102). 3-MC treatment results in absorbance maxima of 450, 449, and 448 nm in the CO-difference spectrum of liver microsomes from DBA/2J, Swiss, and C57BL/6J mice, respectively (128).

b. Effects of Inducers on Enzyme Activity: PB pretreatment in rats induces preferentially ethylmorphine N-demethylase, aniline hydroxylase, and epoxide hydrolase activities (102,106). 3-MC pretreatment of rats preferentially induced benzo[a]pyrene hydroxylase and 7-ethoxycoumarin-O-dealkylase activities, but not ethylmorphine N-demethylase activ-



	Normal	Phenobarbital	Methyl-Cholanthrene	Polychlorinated Biphenyls
Ethylmorphine N-demethylase (μ mol HCHO/mg protein/hr)	0.43	1.59	0.47	1.15
Benzo[a]pyrene Hydroxylase (nmol OHBP/mg protein/hr)	7.50	20.09	58.52	47.94

Fig. 3. Differences in spectral and catalytic properties of liver microsomes from rats pretreated with phenobarbital, 75 mg/kg/day; methylcholanthrene, 25 mg/kg/day; and Aroclor 1254 (Polychlorinated biphenyls), 50 mg/kg/day for 4 days each. Spectra depicted are CO-difference spectra with peak maxima indicated. Reproduced from Alvares et al. (160).

ity.

In mice PB pretreatment induces cytochrome P-450 and associated activities as in rats (128). 3-MC caused no significant induction in N-demethylase activity, but markedly induces benzo[a]pyrene hydroxylase activity in C57 mice (128, 130). In contrast, the DBA strain of mice are relatively "non-responsive" to the inductive effects of 3-MC. The inductive properties of Aroclor 1254 in mice were similar to those elicited by PB in rats (128).

c. Effects of Inducers on Electrophoretic Mobilities: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) has been used to evaluate the purity of an enzyme preparation and to determine the subunit molecular weight of different microsomal preparations (71). The SDS-PAGE pattern of partially purified cytochrome P-450 from PB-treated rats differed considerably in the 47,000 to 50,000 molecular weight region from the pattern observed with partially purified cytochrome P-450 from 3-MC-treated rats (111,112). These protein bands were presumed to contain cytochrome P-450 because of the positive staining with benzidine, which tests for heme-dependent peroxidase activity (73).

G. Ethanol as an Inducer of Drug-Metabolizing Enzymes: Chronic ethanol pretreatment also induces the oxidative metabolism of certain xenobiotics. It increases liver microsomal protein and phospholipids (132), cytochrome P-450 content (89), and the activities of various drug-metabolizing enzymes (40,41,133). These inductive properties of ethanol have been studied primarily in rats and humans. Increased metabolism of xenobiotics can sometimes result in the formation of toxic metabolites. This may well be the effect seen in the observed interactions of ethanol with CCl_4 (134), and acetaminophen (135), to increase hepatotoxicity.

A new isozyme of cytochrome P-450 has been purified to electrophoretic homogeneity from hepatic microsomes of rabbits treated chronically with ethanol (37). This cytochrome showed an unusual high activity for the p-hydroxylation of aniline, when compared to other rabbit liver isozymes. Previous studies by Villeneuve et al. (136) have shown that cytochrome P-450 from ethanol-fed Sprague-Dawley rats showed some substrate specificity differing from cytochrome P-450-mediated oxidations determined in rats pretreated with other prototypic inducers, PB and 3-MC.

H. Epoxide hydrolase: As mentioned above epoxide hydrolase activity was induced by PB pretreatment in rats. Epoxide hydrolase has been induced by a number of other compounds (137-140). However, induction of this enzyme by ethanol has not been demonstrated.

Chemically reactive epoxide metabolites are produced by the oxidation of alkene and arene compounds by the microsomal monooxygenase enzymes (141,142). These epoxides are reactive electrophiles which may be further metabolized to dihydrodiols by epoxide hydrolase (143). Epoxide hydrolase is a microsomal enzyme found in most tissues, with the liver containing a high level of this enzyme (144). A cytosolic epoxide hydrolase has also been characterized in mouse liver (148).

Differences in epoxide hydrolase activity have been shown between inbred strains of mice (145,146). Genetic polymorphism has also been described for this enzyme in mice (147). C57 and A/J mice were representative of the two phenotypes. The mice were separated in the two representative phenotypic classes on the basis of differences in pH optima and heat sensitivity of the microsomal epoxide hydrolase. It was of interest to determine whether there exists a relationship between the

acetylation and epoxide hydrolase polymorphisms and to determine the effects of ethanol as an inducer on these relationships. Some preliminary studies were therefore carried out to determine the ability of ethanol to induce epoxide hydrolase in the strains of mice of interest, and to compare the induction in mice with the induction in rats.

SPECIFIC AIMS

In order to understand the effects of ethanol on acetylation reactions in humans the A/J and C57 acetylator mouse models needed to be adapted to an ethanol-containing liquid diet. Before the present studies began, little information was available on adapting mice to an ethanol-containing diet. Although ethanol had been shown to be a liver microsomal enzyme inducer in rats, the potential effects of ethanol on hepatic drug metabolism in mice had to be determined. Prior to the ethanol experiments, two strains of mice were first characterized for the inductive effects of the classical microsomal enzyme inducers; PB, 3-MC, and PCBs. Once the classical enzyme induction studies were completed, the effects of ethanol as a microsomal enzyme inducer in mice were determined and compared to those observed with the classical inducing agents.

Due to the lack of induction studies in mice, all methodologies were first developed in the Sprague-Dawley and Long-Evans rats. Sprague-Dawley rats are widely used for such studies and hence were an appropriate strain for comparison. This also permitted the comparison of interspecies differences in the regulation of monooxygenases between the two acetylator phenotypic mice and the two strains of rats.

Once the hepatic monooxygenase studies were completed, the effects of acute and chronic ethanol administration on acetylation rates were determined in the mouse model.

Based on this logical scheme, the following approaches were examined:

I. <u>Charaterization and Regulation of the Hepatic Monooxygenase System:</u>

A. The inductive properties of PB, 3-MC, and PCBs in A/J and C57 mice.

B. The inductive properties of PB, 3-MC, and PCBs in Sprague-Dawley and Long-Evans rats.

C. The inductive properties of alcohols in rats and mice.

1. In vivo studies establishing a chronic ethanol-containing liquid diet for rats and mice.
2. Effects of chronic ethanol ingestion on hepatic monooxygenases in rats and mice.
3. The inductive properties of isopropanol in rats.
4. The effects of ethanol on the microsomal epoxide hydrolase activity.

II. Effects of Ethanol on Acetylation Characteristics in A/J & C57 Mice:

A. Development of drug assays for acetylation phenotyping.

B. Effects of acute ethanol administration on rates of acetylation of the various test drugs.

1. Effects of acute ethanol administration on apparent plasma half-life of test drugs.
2. Effects of acute ethanol administration on erythrocyte NAT activity.
3. Effects of acute ethanol administration on urinary excretion of SMZ, SNL, and PA.

C. Effects of chronic ethanol administration on rates of acetylation of the various test drugs.

1. Effects of chronic ethanol administration on erythrocyte NAT activity.
2. Effects of chronic ethanol administration on urinary excretion of SMZ, SNL, and PA.

A. ANIMALS: Male rodents were used for all of the studies presented. Sprague-Dawley rats were purchased from Taconic Farms, Germantown, NY; Long-Evans rats from Blue Spruce Farms, Altamont, NY, and the A/J and C57 mice from Jackson Laboratories, Bar Harbor, ME. Animals were allowed to adjust to conditions in the animal facility for a minimum of 4 days. All were maintained on standard laboratory chow diet (Ralston Purina, St. Louis, MO) or Liquid Ethanol Diet (Bio Serv, Frenchtown, NJ) and water ad lib. Lights were turned on at 0600 hr for a 12 hr period. Rats were killed by decapitation and mice were killed by cervical dislocation 24 hr after the last injection or 12 hr after the last feeding.

B. CHEMICALS: The following drugs and chemicals were purchased from the manufacturers indicated and used without further purification: benzo[a]pyrene, 7-ethoxycoumarin, NADPH, NADP, acetyl-coenzyme A (AcCoA), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, N-acetylprocainamide, and procainamide (Sigma Chemical Company, St. Louis, MO); HPLC columns and solvents (Waters Assoc., Milford, MA); ethylmorphine (Merck Chemical, Rahway, NJ); Aroclor 1254 (Analabs, North Haven, CT); absolute ethanol (U.S. Chemical Company, NY,NY); Ethanol Liquid Diet (Bio Serv Inc., Frenchtown, NJ); sulfamethazine, sodium sulfadiazine (ICN Pharmaceuticals Inc., Plainview, NY); sodium phenobarbital, aniline, and clinical chemistry reagents (J.T. Baker Company, Phillipsburg, NJ); N-(1-naphthyl) ethylenediamine dihydrochloride (Eastman Kodak, Rochester, NY). Electrophoresis materials were purchased from BioRad, Richmond, CA. Gel filtration materials were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. 3-hydroxybenzo[a]pyrene standard was a gift received from Dr. S. Yang, Uniformed Services University, Bethesda, MD.

C. TREATMENT OF ANIMALS:

1. Induction Studies: Animals were injected intraperitoneally with PB, 75 mg/kg/day for 4 days; 3-MC, 25 mg/kg/day for 4 days, or the PCBs mixture, Aroclor 1254, 50 mg/kg/day for 4 days. 3-MC and PCBs were dissolved in corn oil and PB was dissolved in distilled water.

2. Liquid Diet Studies: The liquid diet (BIO-MIX #F711-C ISO-CAL), prepackaged by Bio Serv, was reconstituted every 2-3 days with cold water and kept refrigerated. The formulation was the same as that used by DeCarli and Lieber (75). The mice received the same formulation, with the addition of cellulose fiber to provide adequate stool formation. The ethanol formulation shown contains 35% ethanol-derived calories. The diet was modified to contain zero-35% ethanol-derived calories by changing the amounts of ethanol and maltose dextrin. Animals were paired by weight and divided into two pretreatment groups: control and ethanol-treated. Control animals were pair fed with control diet based on the previous day's diet consumption by the ethanol group of animals. Thus, both control and ethanol-treated animals received isocaloric diets. Ethanol-treated rats received a liquid diet containing 12% ethanol-derived calories on days 1-3, 24% on days 4-5, and 35% on days 6-34. The C57 mice had a high mortality rate, when placed on the original ethanol diet of DeCarli and Lieber which contained 35% ethanol-derived calories. Therefore, a modification of the ethanol treatment regimen developed by Petersen et al. (52) was used. In this modification ethanol-treated mice received a liquid diet containing 10% ethanol-derived calories on days 1-5, 20% on days 6-10, 30% on days 11-21. The modified regimen was well tolerated by the two strains of mice.

3. Acute Ethanol Studies: In the acute ethanol experiments ethanol 3.0 g/kg was administered intraperitoneally. Ethanol was mixed in water and administered in a volume of 20 ml/kg (52). Controls received an equal volume of water. Animals were then injected 30 min later with a challenge dose of SMZ, SNL, or PA (2.5 mg in a volume of 0.5 ml).

D. PREPARATION OF TISSUES AND FLUIDS:

1. Liver Studies: Livers were excised, weighed and homogenized in 4 volumes of 50 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.4), containing 1 mM dithiothreitol, in a glass and teflon homogenizer. In the initial studies where monooxygenase activities were determined, livers were homogenized in 1.15% KCl. Homogenate was centrifuged in a Sorvall RC5-B centrifuge at 9,000 x g for 20 min and an aliquot of the 9,000 x g supernatant was assayed for benzo(a)pyrene hydroxylase activity. The 9,000 x g supernatant was centrifuged in a Beckman L5-50 centrifuge at 105,000 x g for 1 hr to sediment the microsomes. The 9,000 x g and 105,000 x g supernatants served as a sources of liver cytosol NAT. The microsomal pellet obtained was suspended in 0.1 M NaH_2PO_4-K_2HPO_4 buffer (pH 7.4) and assayed for cytochrome P-450 content, ethylmorphine N-demethylase, 7-ethoxycoumarin O-deethylase, and aniline hydroxylase activities.

2. Blood Studies: Animals were placed in plexiglass restrainers, tails clipped, and 50 µl of blood was drawn into a heparinized micropipette. The blood was then hemolysed by pipetting into 0.5 ml distilled H_2O and the hemolysate used for the Bratton-Marshall (61) assay and/or NAT activity.

3. Urine Studies: After the appropriate pretreatment, animals were placed in metabolic cages for the collection of urine. Urine was collected at various times up to 24 hours. At each sampling time the

cage was rinsed with distilled H_2O and the total urine volume brought up to 10 ml.

E. ENZYMIC ASSAYS:

1. Cytochrome P-450: The CO-difference spectrum of reduced microsomes was used to measure cytochrome P-450. Microsomes equivalent to 100 mg of liver, wet weight, were used to assay for cytochrome P-450 content. The hemeprotein content was determined by the method of Omura and Sato using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ between 450 and 500 nm (53). The CO-difference spectrum of dithionite reduced microsomes was recorded using an Aminco DW-2a spectrophotometer equipped with a microproccessor data analyzer.

2. Ethylmorphine N-demethylase: Ethylmorphine is N-demethylated by liver microsomes to form norethylmorphine and HCHO. The HCHO formed, when reacted with Nash's reagent, forms a yellow chromogen, the absorbance of which was measured at 412 nm using a Gilford 250 UV/VIS spectrophotometer. N-demethylase activity was measured as described by Alvares and Mannering (55), using the modified Nash's reagent described by Anders and Mannering (56). N-demethylase activity was determined using liver microsomes equivalent to 100 mg liver, wet weight. The incubation mixture contained 10 µmole $MgCl_2$, 37.5 µmole semicarbazide HCl to trap HCHO, 20 µmole of Na_2HPO_4-KH_2PO_4 buffer, pH 7.4, and a NADPH regenerating system, 2 units of glucose-6-phosphate dehydrogenase, 2 µmole of NADP and 20 µ mole of glucose-6-phosphate. The samples were incubated for 20 min.

3. Benzo[a]pyrene hydroxylase: The reaction mixture contained 3 µmole $MgCl_2$, 1 µmole NADPH, 100 µmole Na_2HPO-KH_2PO_4 buffer pH 7.4, 9,000 x g supernatant equivalent to 4 mg liver, wet weight, and 100 µmole

of benzo(a)pyrene in a total volume of 1.0 ml. The samples were incubated in the dark for 5 min at 37°C. The reaction was stopped by the addition of 1 ml cold acetone. The amount of phenolic metabolites formed was measured as described by Nebert and Gelboin (57). The fluorescence of the products formed was determined using a Perkin Elmer MPF-44A fluorescence spectrophotometer, slit width 6 nm, excitation wavelength of 396 nm and emission wavelength of 522 nm. The fluorescence intensity of the phenolic products formed was determined using 3-hydroxybenzo(a)pyrene as a standard.

4. Aniline hydroxylase: Aniline hydroxylase activity was measured by following the conversion of aniline to p-aminophenol, according to the method of Imai et al. (58). The incubation mixture contained microsomal protein equivalent to 100 mg liver, wet weight, 8 mM aniline, 0.32 mM NADP, 3 mM glucose-6-phosphate, 2.5 mM $MgCl_2$, 1.3 units glucose-6-phosphate dehydrogenase, 75 mM Tris buffer (pH 7.4). The reaction was initiated by the addition of microsomes. After 20 min the reaction was stopped by the addition of 0.5 ml of 20% trichloroacetic acid. After centrifugation for 10 min at 10,000 x g, 1 ml of supernatant was pipetted out and 0.25 ml of 10% Na_2CO_3 was added, followed by 1.25 ml of 2% phenol in 0.2 M NaOH. The resulting blue color was measured at 630 nm using a Gilford 250 Spectrophotometer.

5. 7-Ethoxycoumarin O-deethylase: This assay is based on the O-dealkylation of 7-ethoxycoumarin to the highly fluorescent metabolite 7-hydroxycoumarin. This assay was carried out as described by Jacobson et al. (59). The incubation mixture consisted of microsomes equivalent to 5 mg liver, wet weight, 0.3 μM 7-ethoxycoumarin, 3 mM $MgCl_2$, and a NADPH-generating system consisting of NADP (0.5 μM), glucose-6-phosphate

(5 μM), and glucose-6-phosphate dehydrogenase (2 units), in a final volume of 1.0 ml of 0.05 M Tris-HCl buffer (pH 7.7). The reaction was started by the addition of the NADPH-generating system. Following incubation for 5 min the contents of the incubation flask were extracted once with 5 ml of n-hexane. After extraction, the tubes were centrifuged and the hexane layer was discarded. Five ml of ether was then added to each tube and the contents were shaken for 5 min. Following centrifugation, 3 ml of the ether extract was transfered to another tube and evaporated to dryness under N_2. The residue was dissolved in 2 ml of 0.1 M K_2HPO_4-KH_2PO_4 buffer (pH 7.4) and then the aqueous layer was washed once with 5 ml hexane. The hexane layer was discarded and the samples were then read on a Perkin-Elmer MPF-44A fluorescence spectrophotometer. The excitation and emission wavelengths were set at 338 nm and 458 nm respectively, with the slit width set at 4 nm. The readings obtained were compared to those obtained when 7-hydroxycoumarin was used as a standard.

6. N-acetyltransferase: NAT activity was determined by the procedure of Glowinski and Weber (11), a micro-modification of the diazotization procedure of Bratton and Marshall (61). NAT activity was monitored using PABA as a substrate. The reaction mixtures (90 μl) contained 50 μg protein of the enzyme source, 20 μl of a 10 mM solution of AcCoA, and 20 μl of 0.2 mM substrate solution. Whole blood lysates or liver cytosols (see tissue preparation) were used as enzyme sources. Control tubes contained no AcCoA. The reaction was stopped by the addition of 10% trichloroacetic acid (w/v) after 5 min. The free drug remaining was diazotized by the addition of sodium nitrite. Excess nitrite was destroyed by the addition of ammonium sulfamate, and

the diazotized product was coupled by addition of N-(1-naphthyl) ethylenediamine dihydrochloride. The chromogen formed was then read at 540 nm against a water blank using a Gilford 250 spectrophotometer.

7. Epoxide Hydrolase: Epoxide hydrolase assays were performed by the Department of Biochemistry and Drug Metabolism, Hoffman-La Roche, Inc., Nutley, New Jersey under the direction of Mr. W. Levin.

Epoxide hydrolase activity of rat and mouse hepatic microsomes was determined by the method of Jerina et al. (60), a sensitive radiometric assay using labeled benzo[a]pyrene 4,5-oxide as a substrate. Microsomes were prepared as previously described, suspended in 0.25 M sucrose (20 mg protein/ml), and stored at -90° for 3-7 days prior to use. The incubation mixture consisted of resuspended microsomes (15 µg), 25 µl of 0.5 M Tris-HCl buffer (pH 8.7), 50 µl of water and and 5 µl of benzo[a]pyrene 4,5-oxide (5.9 µCi/µmol), in that order to reach a final volume of 80 µl. The reaction mixture was incubated for 5 min and terminated by the addition of 25 µl of tetrahydrofuran. After the incubation, 35 µl of the mixture was applied to a 5 x 20 cm LQDF silica gel thin-layer plate. After development of the plates, the product bands were localized under ultraviolet light and scraped into scintillation vials containing 1.0 ml methanol. After addition of 15 ml Scintisol, radioactivity was measured by scintillation spectrometry.

F. CHEMICAL ASSAYS:

1. Determination of SMZ, SNL and Acetylated Metabolities in Urine: These compounds were quantified by a HPLC procedure utilizing a Waters Associates Liquid Chromatograph. Aliquots of urine (10 µl) from *in vivo* experiments were added to 1.0 ml water in 10 x 75 mm test tubes.

An aliquot of the internal standard solution, SDZ (50 µg/ml), was added and mixed well. An aliquot (20 µl) of this solution was injected into a Waters HPLC System. This system was equipped with solvent delivery models M510 and M45, a M610 system controller, a Rheodyne injector with a 20 µl loop, a Model 441 absorbance detector at 254 nm, and a Hewlett Packard 3390 integrator/plotter. Separation was carried out on a C-18 5 µm RAD-PAK column. The column was eluted with 10 mM sodium acetate buffer/methanol (68:32) at a rate of 1.0 ml/min for the separation of SNL and metabolite, and at a rate of 2.0 ml for SMZ and metabolites.

2. Determination of apparent plasma half life of SMZ and SNL: These compounds were determined by a modified method of Bratton and Marshall (61). Blood (0.05 ml) was drawn from each animal at 1, 3, 5 and 8 hr and added to 1.50 ml water and 0.50 ml trichloracetic acid (10% w/v), mixed and centrifuged. Two 0.75 ml aliquots of the supernatant were transfered to glass test tubes. HCl (0.10 ml, 4 N) was added to one tube and in a heating block (100° C) for 1 hr and cooled to determine total drug concentration. Sodium nitrite solution (0.10 ml, 1 g/l) was added to both tubes and 3 min later 0.10 ml of ammonium sulfamate solution (5 g/l) was added. After 3 min, 0.50 ml of N-(1-naphthyl) ethylenediamine dihyrochloride solution (0.5 g/l) was added and the samples were read using a Gilford UV-VIS Spectrophotometer set at 540 nm.

3. HPLC Determination of Procainamide and N-Acetylprocainamide: These compounds were quantified by a HPLC procedure similar to that developed by Carr et al. (63) and Pang et al. (63). Urine or blood samples (50 µl) from *in vivo* experiments were pipetted into 16 x 100 mm glass test tubes. To these tubes 1.0 ml of distilled H_2O and 0.2 ml 1N NaOH were added and mixed well. Following these additions 6.0 ml of chloroform

was added, the tubes vortexed and centrifuged at 3,000 x g for 5 min. After centrifugation the aqueous phase (top layer) was suctioned off and a 4.0 ml aliquot of the organic phase was transfered to polypropylene tubes and placed under a stream of nitrogen in a Myer analytical evaporator set at 50° C. The residue was reconstituted with 50-200 µl of the mobile phase. An aliquot (20 µl) of this solution was injected into the Waters HPLC System. Separation was carried out on a C-18 5 µm RAD-PAK column. The column was eluted at a rate of 1.0 ml/min with sodium acetate: acetic acid:water: acetonitrile:methanol: 2.5/10/500/676/41, w/v/v/v/v.

4. Protein determination: Protein content of the various cell fractions was determined by the method of Lowry et al. (70) using bovine serum albumin as a standard.

5. Separation of microsomal proteins by polyacrylamide gel electrophoresis: SDS-PAGE was conducted using the system of Laemmli (71). The stacking gel contained 3% acrylamide and the separation gel (11 x 8.2 x 0.27 cm) contained 10% acrylamide. Electrophoresis was carried out at 7°C at 10 and 20 mA/gel during stacking and separation, respectively, until the tracking dye, bromophenol blue, reached the bottom of the slab. The electrophoresis took about 7 hr. The gel was stained for protein, as described by Fairbanks et al. (72), except that the staining solution contained 0.25% Coomassie blue. Heme-associated peroxidase activity was determined by the method of Thomas et al. (73). Gels were scanned immediately after electrophoresis, using a LKB Zeineth soft laser scanning densitometer.

G. HISTOPATHOLOGIC EXAMINATION OF TISSUES: The tissues were prepared by Dr. D. Patrick, Diagnostic Services and Comparative Medicine,

and examined by Dr. M. T. Smith, Department of Pathology, located at Uniformed Services University, Bethesda, MD.

Fresh frozen renal sections were prepared to determine if crystals of SMZ or AcSMZ were present after drug treatment. Tissues were embedded in Tissue-Tek II, O.C.T. Compound Embedding Medium. Frozen sections were cut (10 μm) on a Damon/IEC Minotome, and were examined unstained under polarized light for birefringent crystals.

Liver tissue was examined for fatty infiltration and cellular damage. Tissue was fixed in 10% neutral buffered formalin. The tissues were then dehydrated and embedded in paraffin. Embedded liver tissue was sectioned on a Leitz 1512 microtome to 4 μm and stained with hemotoxylin and eosin, and examined with a light microscope.

H. STATISTICS: The statisical significance of differences between control and treated groups was evaluated by Student's t test. A p value of 0.05 or less was considered as statistically significant.

I. CHARACTERIZATION AND REGULATION OF HEPATIC MONOOXYGENASE SYSTEM:

A. The inductive properties of PB, 3 MC, and PCBs in A/J and C57 mice: The effects of pretreatment of C57 and A/J mice by PB, 3-MC, or Aroclor 1254, on hepatic cytochrome P-450 content (Fig. 4), on ethylmorphine N-demethylase (Fig. 5), and on benzo[a]pyrene hydroxylase (Fig. 6) activities were determined. PB pretreatment resulted in significant increases in cytochrome P-450 content in both strains of mice. 3-MC caused a 3-fold increase in cytochrome P-450 content in the C57 strain, but no significant increase in cytochrome P-450 content in livers of A/J mice. PCBs caused a significant increase in the hemeprotein content in both strains of mice. Ethylmorphine N-demethylase activities in livers of untreated and variously treated mice were assayed and results are shown in Fig. 5. A/J mice were induced to a greater extent than the C57 mice by PB. 3-MC had little effect as an inducer of ethylmorphine N-demethylase activities in livers from both strains of mice. Benzo[a]-pyrene hydroxylase activities in controls and variously treated mice were assayed and results are shown in Fig. 6. PB caused slight, but statistically significant increases in the hydroxylase activities in both strains of mice. As expected from studies of Nebert and Gielen (130), 3-MC caused a marked, 8-fold increase in benzo[a]pyrene hydroxylase activity in the C57 mice. 3-MC also induced the A/J mice, but to a much lesser extent. The inductive properties of PCBs were intermediate between the inductive properties of PB and 3-MC. A comparison of the basal monooxygenase activities between the two strains indicates that

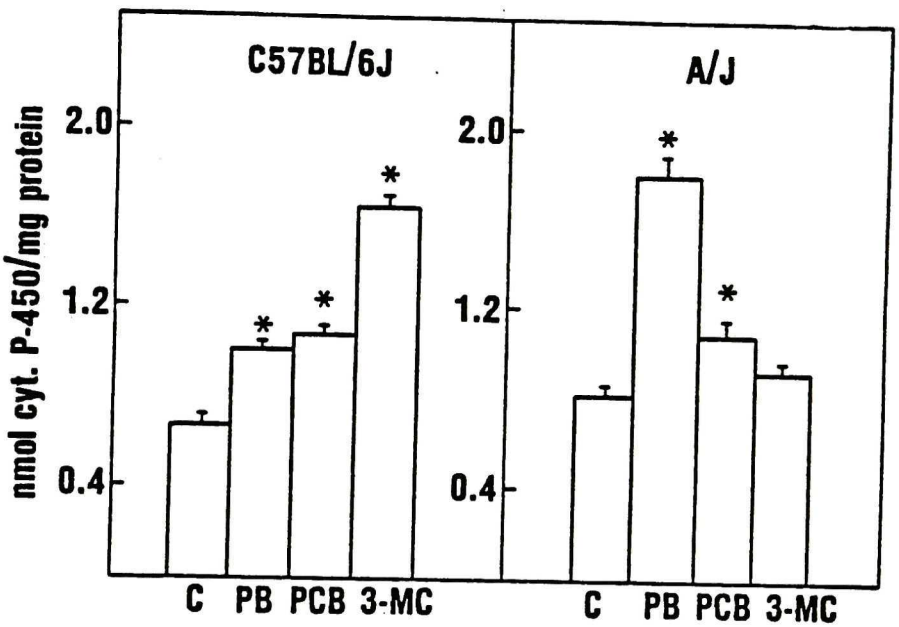


Fig. 4. Effects of phenobarbital (PB), polychlorinated biphenyls (PCB), and 3-methylcholanthrene (3-MC) on hepatic cytochrome P-450 content. Mice were pretreated with PB, 75 mg/kg/day, PCB (Aroclor 1254), 50 mg/kg /day, 3-MC, 25 mg/kg/day, for 4 days by ip. injection. Mice were killed 24 hr after last injection. Each bar represents the mean ± S.E. for at least 5 animals. Asterisk represents values significantly different from the respective control value ($p < 0.05$).

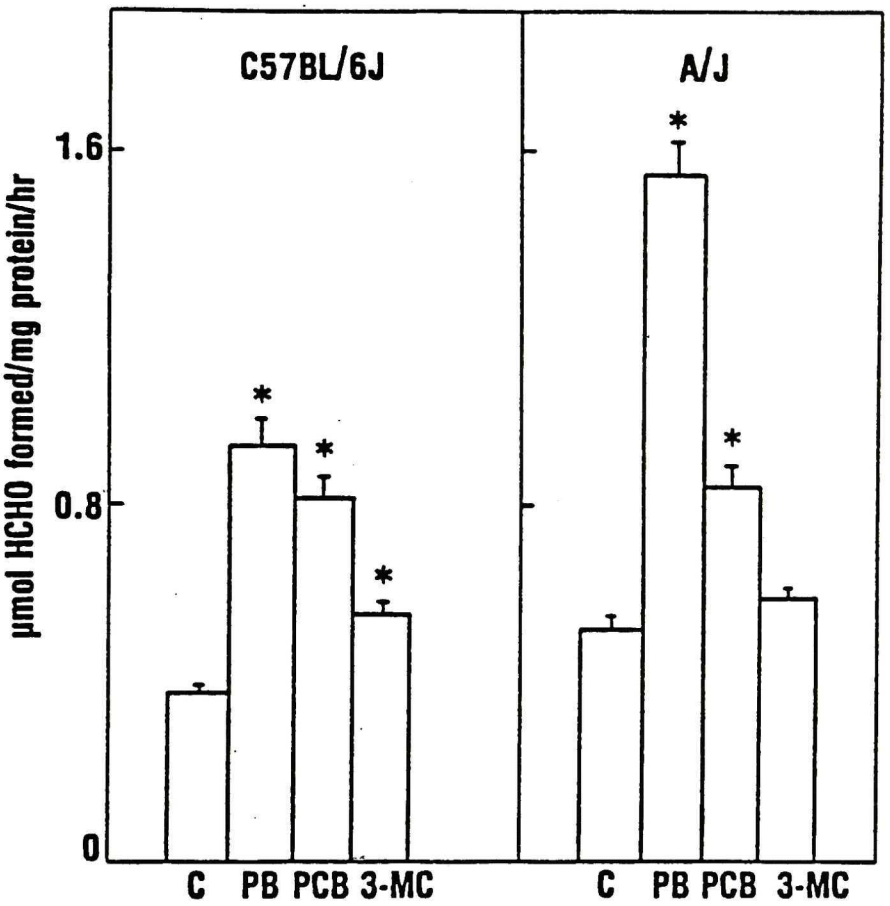


Fig. 5. Effects of phenobarbital (PB), polychlorinated biphenyls (PCB), and 3-methylcholanthrene (3-MC) on hepatic ethylmorphine N-demethylase activities. Mice were pretreated with compounds as described in legend to Fig. 4. Each bar represents the mean ± S.E. for at least 5 animals. Asterisk represents values significantly different from the respective control value ($p < 0.05$).

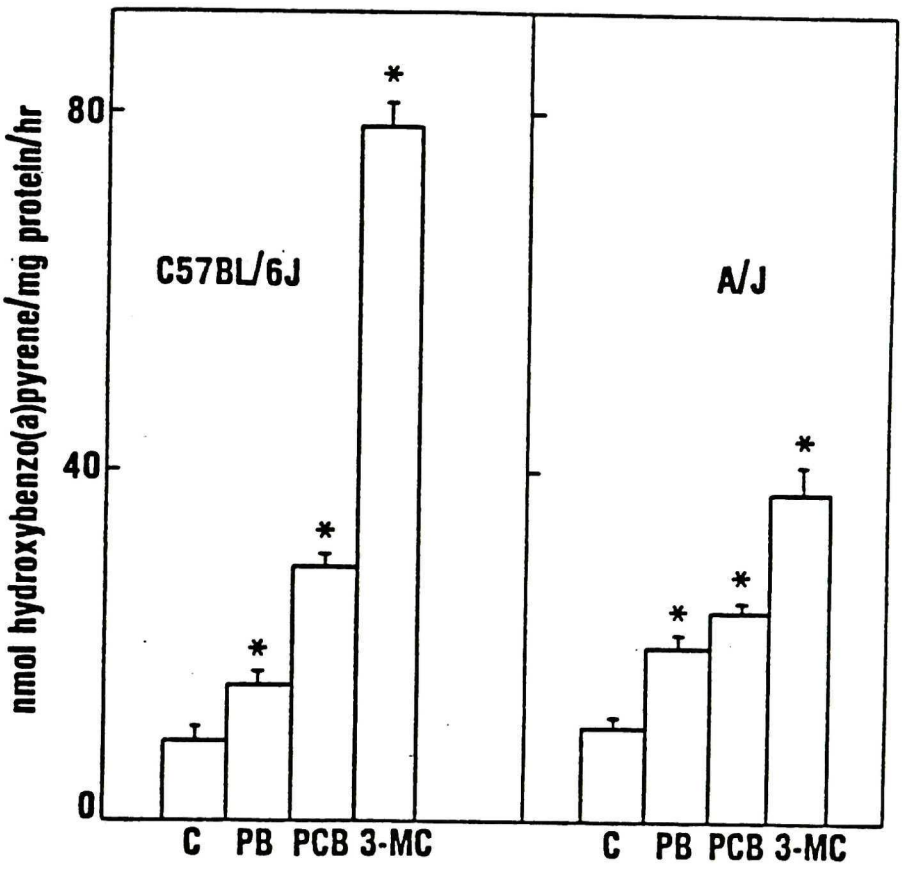


Fig. 6. Effects of phenobarbital (PB), polychlorinated biphenyls (PCB), and 3-methylcholanthrene (3-MC) on hepatic benzo[a]pyrene hydroxylase activities. Mice were pretreated as described in legend to Fig. 4. Each bar represents the mean ± S.E. for at least 5 animals. Asterisk represents values significantly different from the respective control value ($p < 0.05$).

the activities are similar in the A/J and C57 mice strains. No significant differences were noted between the strains for benzo[a]pyrene hydroxylase activity (Fig. 6).

In the above studies the effects of three inducers; PB, 3-MC and PCBs on cytochrome P-450 content and associated enzymic activities were measured. Basal monooxygenase activities were similar in the two strains. The administration of the inducers PB and PCBs resulted in significant increases in cytochrome P-450 content and ethylmorphine N-demethylase activity with minimal changes in benzo[a]pyrene hydroxylase activity. 3-MC increased benzo[a]pyrene hydroxylase activity to a greater extent in the C57 mice than the A/J mice. 3-MC had minimal effect on ethylmorphine N-demethylase activity in either strain of mice.

CO-difference spectra of liver microsomes from the variously pretreated mice were determined (Fig. 7). Absorbance maxima of the CO-difference spectra occurred at 450 nm determined from microsomes prepared from livers of untreated, PB, and PCBs pretreated mice. For the C57 mice the CO-difference spectra absorbance maximum occured at 449 nm, whereas in the A/J mice, the absorbance maximum occured at 450 nm after 3-MC pretreatment. Thus, in the A/J mice the "minimal responsiveness" to 3-MC induction of aryl hydrocarbon (benzo[a]pyrene) hydroxylase activities is reflected by a lack of change in spectral properties of the cytochrome induced. The induction of cytochrome P-449 in the 3-MC "responsive" C57 strain of mice was substantiated in these studies.

The electrophoretic mobilities, as determined by SDS-PAGE, of the solubilized microsomal preparations utilized to study the enzymic activties of C57 and A/J mice treated with the various inducers were determined (Fig. 8). Those treatments which induced cytochrome P-450 showed more

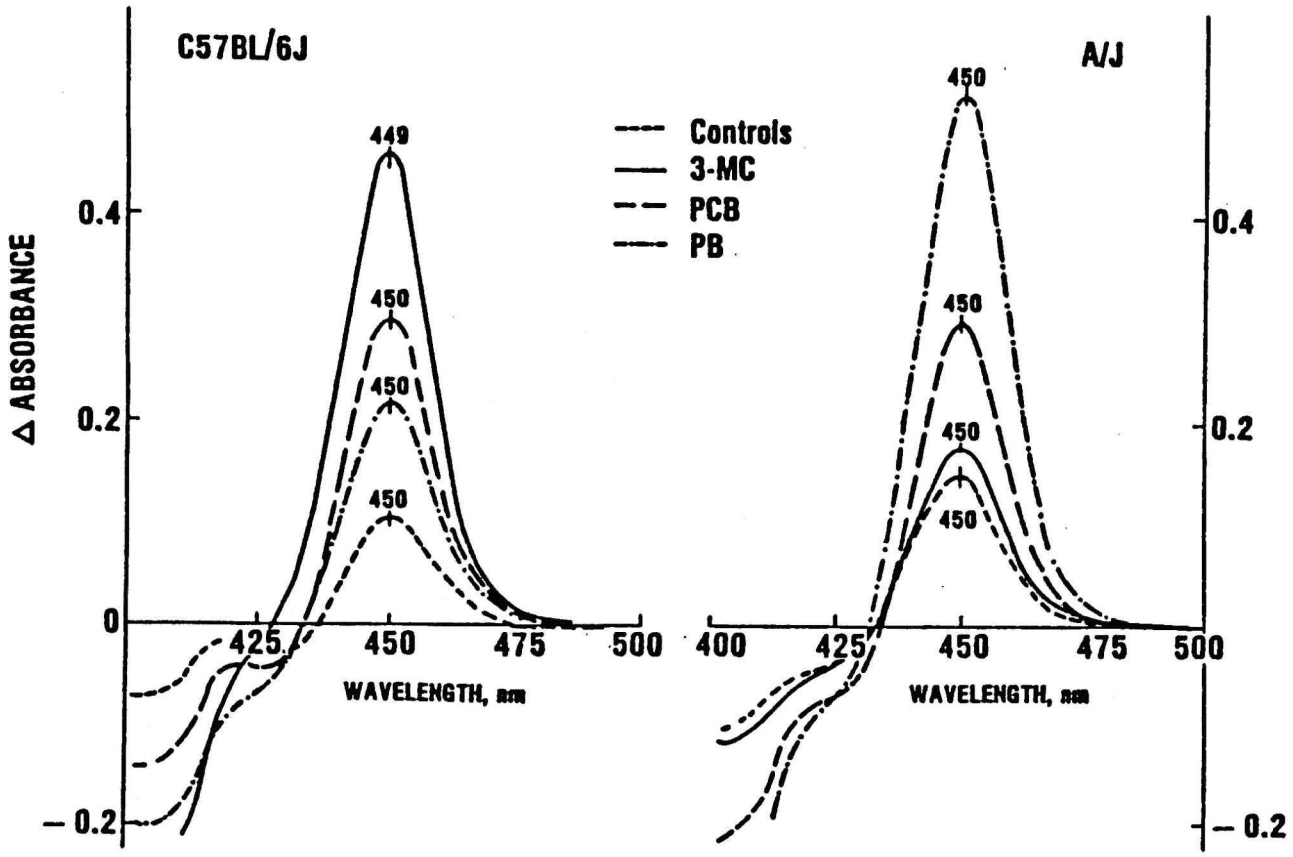


Fig. 7. CO-difference spectra of liver microsomes from untreated, phenobarbital (PB), polychlorinated biphenyls (PCB), and 3-methylcholanthrene (3-MC)-treated mice. Liver microsomal suspensions were prepared, reduced with dithionite and placed in reference and sample cuvettes. CO was then bubbled through the sample cuvette, and the difference spectra was recorded.

intense staining for proteins associated with cytochromes P-450 in the molecular weight range of 53,000-60,000. This included the preferential induction by 3-MC and PCBs in the C57 mice, and PB and PCBs in the A/J mice. The most striking feature of the protein staining bands from 3-MC treated mice was the pronounced staining of a band at about 55,000 molecular weight (well B). This band has previously been shown to be associated with cytochrome P-448 (77). Staining in this region was minimal when microsomes from 3-MC treated A/J mice were used (well F). The ability of PB to preferentially induce cytochrome P-450 in the A/J mice, and of 3-MC to preferentially induce cytochrome P-449 in the C57 mice, was confirmed when the electrophoretic bands in the P-450 region were stained for heme peroxidase activity (Fig. 9). The above studies provide evidence that in the two strains of mice the basal hepatic monooxygenase activities are not correlated with acetylation phenotype. There appeared to be no apparent differences in the basal monooxygenase activities between the fast (C57) acetylator and the slow (A/J) acetylator mouse models.

The novel findings from these experiments were that the fast acetylators were highly responsive to the inducing properties of 3-MC; whereas, the slow acetylators were relatively much less responsive to the inducing properties of the polycyclic hydrocarbon. The latter strain appeared to be more responsive to the inducing properties of the PB class of inducers. Thus, in addition to showing acetylator polymorphism, the two strains of mice showed differences in response to the inducing effects of PB and 3-MC as reflected in the inducibility of cytochromes P-450 and the associated enzymatic activities in the liver.

B. <u>The inductive properties of PB, 3-MC, and PCBs in Sprague-</u>

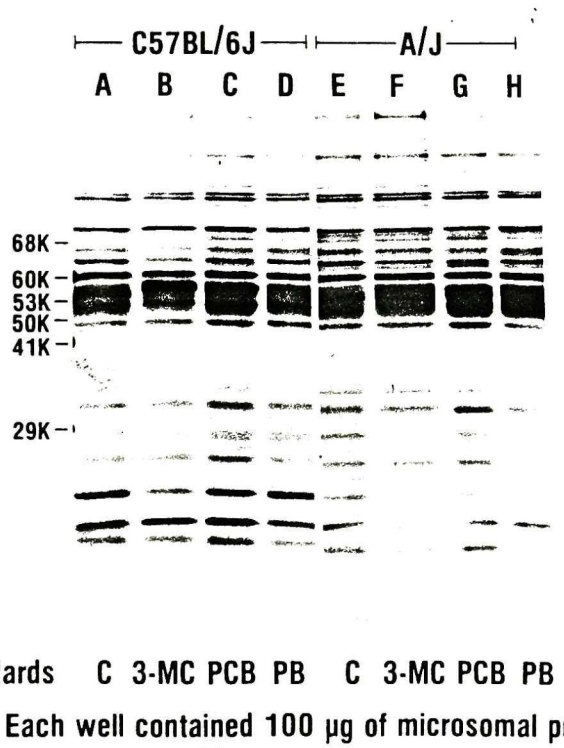


Fig. 8. SDS-PAGE of liver microsomes from untreated, 3-MC, PCB, and PB-treated mice. Microsomal suspensions containing 100 µg of protein were treated with SDS and subjected to slab gel electrophoresis, as described in the Methods section. Protein standards with known molecular weights: bovine serum albumin, 68,000; catalase, 60,000; glutamate dehydrogenase, 53,000; fumarase, 50,000; alcohol dehydrogenase, 41,000; and carbonic anhydrase, 29,000.

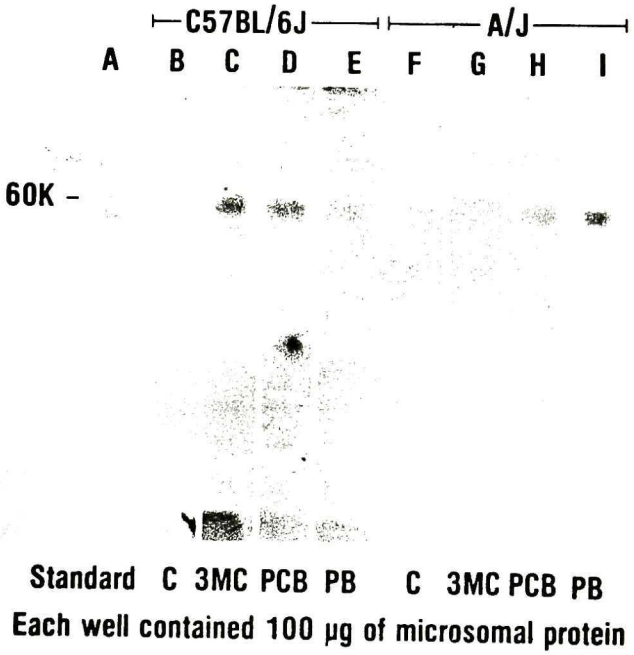


Fig. 9. Staining of peroxidase activity of liver microsomes from untreated, 3-MC, PCB, and PB-treated mice. Microsomal suspensions containing 100 µg protein were subjected to slab gel electrophoresis as described in the Methods section. The gel was stained for peroxidase activity by the method of Thomas et al. (73). Well A contained catalase, with a known molecular weight of 60,000.

Dawley and Long-Evans rats: Sprague-Dawley and Long-Evans rats were used to study the effects of the classical inducers in rats. A study by Ryan et al. (76), indicated that there might be slight differences in the primary structure of hepatic cytochromes P-450 in different strains of rats. A major objective of the rat studies reported here was to indirectly verify previously reported data by Alvares' (99-103) and other laboratories; and to directly verify my experimental techniques. A second major objective was to determine if previously reported differences in the primary structure of cytochromes P-450 by Ryan et al. (76) were reflected in the response of these two strains of rats to the inducers which were used in the mice studies, ie. PB, 3-MC and PCBs. The inducers, dosages and routes of administration were similar to those used previously by other investigators.

The effects of PB, 3-MC, and PCBs on hepatic microsomal monooxygenases were determined in the two strains of rats (Tables 7 and 8). In summary, all three inducers tested increased cytochrome P-450 content of liver microsomes. PB preferentially induced ethylmorphine N-demethylase activity in both strains of rats. The absorbance maximum of the CO-difference spectra of reduced liver microsomes from untreated and PB-treated rats occurred at 450 nm. 3-MC preferentially induced benzo[a]-pyrene hydroxylase activities and caused a spectral shift in the CO-difference spectrum of reduced microsomes to 448 nm in both strains of rats. PCBs showed a "mixed" type of induction, possessing in rats the inductive properties of both PB and 3-MC classes of inducers.

There were minimal differences in the inductive properties between the two strains of rats. Thus, these data obtained with rats showed that although mice demonstrate strain differences in response to

Table 7: Effects of phenobarbital (PB), Aroclor 1254 (PCBs), and 3-methylcholanthrene (3-MC) on cytochrome P-450 content, and associated enzyme activities in Sprague-Dawley rats[a]

Assay	Controls	PB	PCBs	3-MC
Liver to body wt. ratio, mg liver/g body wt.	46.7 ± 1.5	58.3[b] ± 1.5	50.2 ± 2.5	52.9[b] ± 2.6
Microsomal protein, mg/g wet wt.	20.9 ± 0.3	27.9[b] ± 1.0	32.9[b] ± 1.6	22.2 ± 0.9
Cytochrome P-450 content, nmoles/mg protein	0.60 ± 0.03	1.69[b] ± 0.09	1.63[b] ± 0.12	1.53[b] ± 0.05
CO-difference spectrum, absorption maximum, nm	450	450	449	448
Ethylmorphine N-demethylase, µmol HCHO/mg protein/hr	0.33 ± 0.02	0.87[b] ± 0.08	0.87[b] ± 0.08	0.33 ± 0.01
Benzo[a]pyrene hydroxylase, nmol OHBP/mg protein/hr	3.8 ± 0.25	7.8[b] ± 0.5	36.7[b] ± 3.1	27.8[b] ± 0.9

[a] Rats were treated with the various inducer compounds at dosages given in legend to Fig. 4. Each value represents mean ± S.E. for 5 animals.

[b] Value significantly different from the respective control value ($p < 0.05$)

Table 8: Effects of phenobarbital (PB), Aroclor 1254 (PCBs), and 3-methylcholanthrene (3-MC) on cytochrome P-450 content, and associated enzyme activities in Long Evans Rats[a]

Assay	Controls	PB	PCBs	3-MC
Liver to body wt. ratio, mg liver/g body wt.	47.3 ± 0.3	50.7 ± 2.3	61.0[b] ± 1.6	56.7[b] ± 2.3
Microsomal protein, mg/g wet wt.	29.3 ± 1.7	26.2 ± 2.2	30.3 ± 1.5	28.2 ± 0.7
Cytochrome P-450 content, nmol/mg protein	0.63 ± 0.02	1.59[b] ± 0.04	1.68[b] ± 0.06	1.03[b] ± 0.04
CO-difference spectrum, absorption maximum, nm	450	450	449	448
Ethylmorphine N-demethylase, µmol HCHO/mg protein/hr	0.18 ± 0.01	1.34[b] ± 0.02	0.63[b] ± 0.04	0.16 ± 0.01
Benzo[a]pyrene hydroxylase, nmol OHBP/mg protein/hr	2.90 ± 0.40	8.9[b] ± 0.3	24.0[b] ± 1.6	20.5[b] ± 2.4

[a] Rats were treated with the various inducer compounds at dosages given in legend to Fig. 4. Each value represents mean ± S.E. for 5 animals.

[b] Value significantly different from the respective control value ($p < 0.05$).

the classical enzyme inducers, such differences were as marked as in the two strains of rats studied here.

C. The inductive properties of alcohols in rats and mice.

1. In vivo studies establishing a chronic ethanol-containing liquid diet for rats and mice: Male Sprague-Dawley and Long-Evans rats were adapted, over the initial 5-day experimental period, from a liquid diet containing 12% ethanol to a diet containing 35% ethanol. The rats were then maintained on the 35% ethanol diet for about 4 weeks. The latter diet was well tolerated by the rats. However, body weights of the rats on the ethanol diet were significantly lower than those on the control liquid diet (Fig. 10). The livers of the treated rats expressed per gram of body weight were larger, but showed no significant changes in microsomal protein contents (Table 9). Gross examination revealed fatty infiltration of the livers taken from the ethanol-treated animals. Histopathology indicated diffuse hepatocellular vascular change characteristic of fatty infiltration.

Of the two strains of mice used in the present studies, the C57 strain appeared to be rather intolerant to long-term treatment with the 35% ethanol-containing diet. In data not shown, a high mortality rate was observed in this strain of mice during the last week of the ethanol-treatment regimen. In marked contrast, no deaths occurred in the A/J mice under these experimental conditions. Due to this strain difference in alcohol tolerance, the ethanol treatment regimen for the mice was modified. The modification involved feeding a 30% ethanol diet during the last 10 days of the experimental period. Details of this protocol are described in the Methods section. The two strains of mice tolerated this regimen well, and no deaths occurred during the 3-week

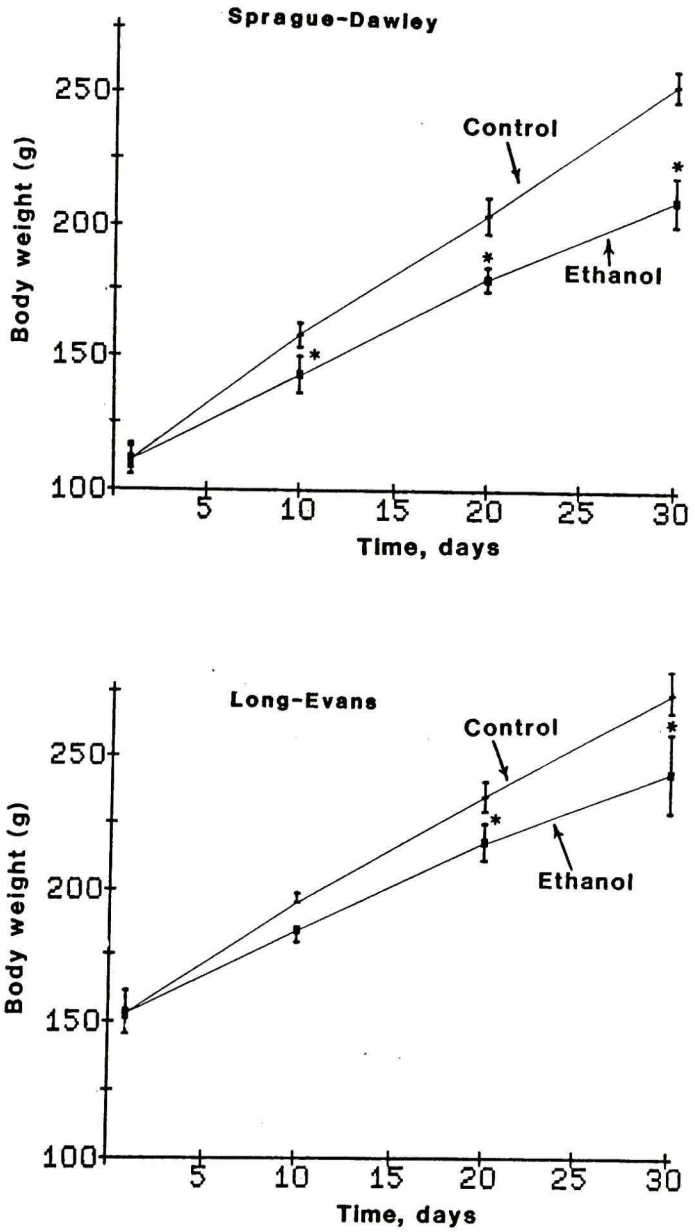


Fig. 10. Body weights of Sprague-Dawley and Long-Evans rats maintained on liquid diets for 34 days. Controls received an isocaloric diet with maltose-dextrin substituted for ethanol-derived calories. Each value represents mean ± S.E. for 5 animals. Asterisk indicates values significantly different from respective control values ($p < 0.05$).

Table 9. Body and liver weights, and microsomal protein contents of animals chronically treated with ethanol[a]

Species/Strain Treatment	Body weight (g)	Liver weight (mg/g body wt)	Microsomal protein (mg/g liver, wet wt)
Rat/ Sprague-Dawley			
Control	254.0 ± 5.9	40.7 ± 1.0	14.3[b] ± 0.4
Ethanol	210.0[c] ± 9.2	52.5[c] ± 2.3	16.3[b] ± 1.3
Rat/ Long-Evans			
Control	276.2 ± 5.7	40.1 ± 1.2	10.5[b] ± 0.3
Ethanol	244.0[c] ± 14.7	42.3 ± 1.0	10.9[b] ± 0.7
Mouse/ C57			
Control	23.0 ± 0.3	53.5 ± 0.4	19.6 ± 1.3
Ethanol	21.6[c] ± 1.2	62.9[c] ± 1.1	29.0[c] ± 0.5
Mouse/ A/J			
Control	23.7 ± 1.1	46.6 ± 1.9	20.6 ± 1.4
Ethanol	18.4[c] ± 1.1	50.3[c] ± 0.6	26.6[c] ± 0.7

[a] Rats and mice were fed the ethanol-containing liquid diet for 34 and 21 days, respectively. Controls received an isocaloric diet with maltose-dextrin substituted for ethanol derived calories. Each value represents mean ± S.E. for 5 animals.

[b] Microsomes used were washed once.

[c] Value significantly different from respective control values ($p < 0.05$).

experimental period.

Unlike rats, mice on the control liquid diets maintained an adult weight of 20-25 g, whereas rats gained 3-4 g/day while on the liquid diets. In data not presented, it was noted that the weight gain in rats on the liquid diet is slightly lower than the weight gain observed with rats fed the normal laboratory chow diet.

As with the rat strains studied, the mean body weights of the two mouse strains fed the ethanol diet were significantly lower than the mean body weights of mice fed the control liquid diet (Fig. 11). Liver weights of the treated mice, expressed as per g of body weight, were significantly greater than the mean liver weights of the respective control strains of mice (Table 9). Less fatty infiltration was apparent in livers of ethanol-treated mice than in livers of ethanol-treated rats. Liver microsomal protein content of livers from ethanol-treated mice were significantly greater than those of livers of mice fed the control liquid diets (Table 9).

2. Effects of chronic ethanol ingestion on hepatic monooxygenases in rats and mice: The effects of chronic ethanol treatment of the four rodent strains on microsomal cytochrome P-450 content and metabolism of 7-ethoxycoumarin, aniline, ethylmorphine and benzo(a)pyrene are presented in Tables 10-13. Cytochrome P-450 contents increased by 55 and 76% in livers of ethanol-treated Sprague-Dawley and Long-Evans rats, respectively (Tables 10 and 11). Cytochrome P-450 was increased to a greater extent in livers of mice fed ethanol. Ethanol-treatment of C57 and A/J mice resulted in a 2- to 3-fold increase in hepatic microsomal cytochrome P-450 levels (Tables 12 and 13). An inspection of the CO-induced difference spectra of the dithionite-reduced liver microsomal

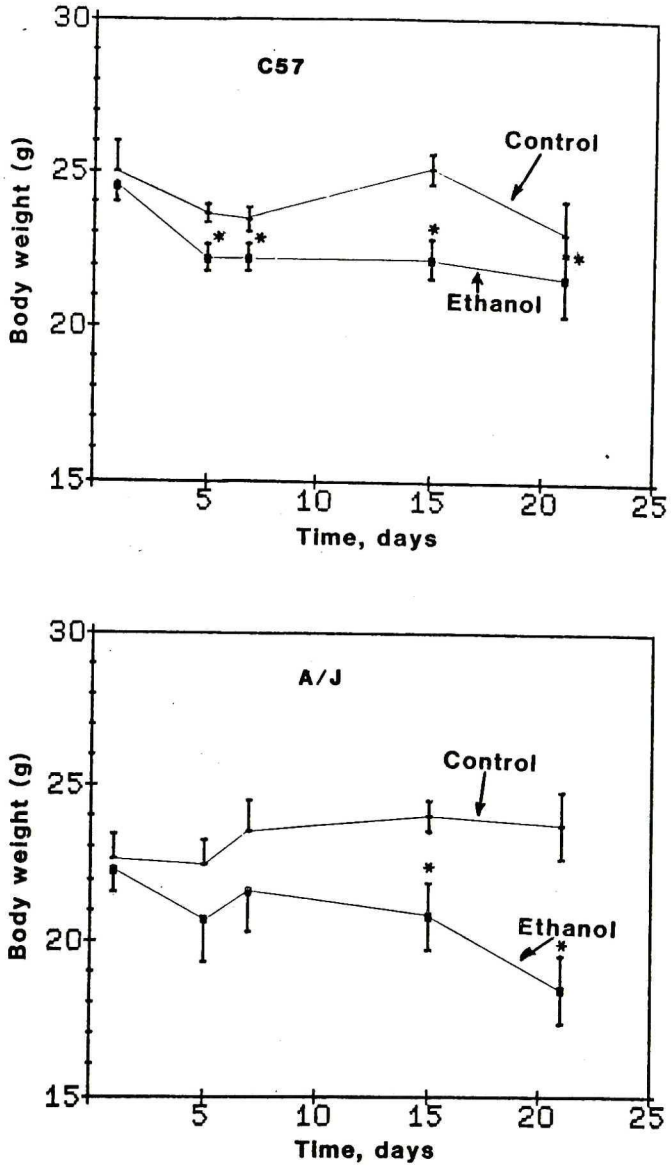


Fig. 11. Body weights of C57 and A/J mice maintained on liquid diets for 21 days. Control received an isocaloric diet with maltose-dextrin substituted for ethanol-derived calories. Each value represents mean ± S.E. for 5 animals. Asterisk indicates values significantly different from respective control values ($p < 0.05$).

Table 10. Comparison of changes in liver microsomal enzymes of Sprague-Dawley rats induced by chronic ethanol treatment[a]

Assay	Control liquid diet	Ethanol liquid diet
Cytochrome P-450 content, nmol/mg protein	0.66 ± 0.03	1.02[b] ± 0.09
CO-difference spectrum absorbance maximum, nm	450	451
Ethoxycoumarin O-deethylase, nmol 7-OH coumarin/mg protein/min	0.93 ± 0.14	2.87[b] ± 0.36
Aniline hydroxylase, nmol p-aminophenol formed/ mg protein/h	48.6 ± 2.0	154.0[b] ± 10.0
Ethylmorphine N-demethylase, µmol HCHO formed/mg protein/h	0.41 ± 0.02	0.46 ± 0.06
Benzo[a]pyrene hydroxylase, nmol OHBP formed /mg protein/h	5.79 ± 0.59	6.52 ± 0.40

[a] Rats were fed the ethanol-containing liquid diet for 34 days. Controls received an isocaloric liquid diet, with maltose-dextrin substituted for ethanol-derived calories. Except for the absorbance data, each value represents mean ± S.E. for 5 animals.

[b] Value significantly different from the respective control value ($p < 0.05$).

Table 11. Comparison of changes in liver microsomal enzymes of Long-Evans rats induced by chronic ethanol treatment[a]

Assay	Control liquid diet	Ethanol liquid diet
Cytochrome P-450 content, nmol/mg protein	1.16 ± 0.03	2.04[b] ± 0.08
CO-difference spectrum absorbance maximum, nm	450	451
Ethoxycoumarin O-deethylase, nmol 7-OH coumarin/mg protein/min	0.49 ± 0.04	1.58[b] ± 0.07
Aniline hydroxylase, nmol p-aminophenol formed/ mg protein/h	30.6 ± 1.5	106.4[b] ± 4.6
Ethylmorphine N-demethylase, µmol HCHO formed/mg protein/h	0.55 ± 0.04	0.41[b] ± 0.01
Benzo[a]pyrene hydroxylase, nmol OHBP formed /mg protein/h	11.13 ± 0.70	8.01[b] ± 0.64

[a] Rats were fed the ethanol-containing liquid diet for 34 days. Controls received an isocaloric liquid diet, with maltose-dextrin substituted for ethanol-derived calories. Except for the absorbance data, each value represents mean ± S.E. for 5 animals.

[b] Value significantly different from the respective control value ($p < 0.05$).

Table 12. Comparison of changes in liver microsomal enzymes of C57 mice induced by chronic ethanol treatment[a]

Assay	Control liquid diet	Ethanol liquid diet
Cytochrome P-450 content, nmol/mg protein	0.62 ± 0.03	1.81[b] ± 0.31
CO-difference spectrum absorbance maximum, nm	450	452
Ethoxycoumarin O-deethylase, nmol 7-OH coumarin/mg protein/min	1.67 ± 0.13	3.67[b] ± 0.23
Aniline hydroxylase, nmol p-aminophenol formed/ mg protein/h	98.7 ± 4.8	185.0[b] ± 4.5
Ethylmorphine N-demethylase, µmol HCHO formed/mg protein/h	0.47 ± 0.04	0.30[b] ± 0.01
Benzo[a]pyrene hydroxylase, nmol OHBP formed /mg protein/h	8.34 ± 0.73	19.3[b] ± 2.2

[a] Mice were fed the ethanol-containing liquid diet for 21 days. Controls received an isocaloric liquid diet, with maltose-dextrin substituted for ethanol-derived calories. Except for the absorbance data, each value represents mean ± S.E. for 5 animals.

[b] Value significantly different from the respective control value ($p < 0.05$).

Table 13. Comparison of changes in liver microsomal enzymes of A/J mice induced by chronic ethanol treatment[a]

Assay	Control liquid diet	Ethanol liquid diet
Cytochrome P-450 content, nmol/mg protein	0.41 ± 0.03	1.08[b] ± 0.01
CO-difference spectrum absorbance maximum, nm	450	452
Ethoxycoumarin O-deethylase, nmol 7-OH coumarin/mg protein/min	1.42 ± 0.06	5.84[b] ± 0.48
Aniline hydroxylase, nmol p-aminophenol formed/ mg protein/h	86.0 ± 7.6	166.4[b] ± 7.6
Ethylmorphine N-demethylase, µmol HCHO formed/mg protein/h	0.24 ± 0.02	0.32[b] ± 0.01
Benzo[a]pyrene hydroxylase, nmol OHBP formed /mg protein/h	6.34 ± 0.34	3.95[b] ± 0.26

[a] Mice were fed the ethanol-containing liquid diet for 21 days. Controls received an isocaloric liquid diet, with maltose-dextrin substituted for ethanol-derived calories. Except for the absorbance data, each value represents mean ± S.E. for 5 animals.

[b] Value significantly different from the respective control value ($p < 0.05$).

suspensions revealed a consistent shift in the absorbance maxima towards longer wavelengths, 451-452 nm in the Soret region, in all four rodent strains receiving ethanol (Tables 10-13). The mouse strains showed a greater shift in the absorbance maxima than the rat strains.

Although the total cytochrome P-450 contents of the ethanol-treated rats and mice were significantly increased, the induction of cytochrome P-450-dependent enzymic activities appeared to be selective. Unlike data obtained when rats were pretreated with the inducers PB, 3-MC or PCBs, in the Sprague-Dawley and Long-Evans rats ethanol treatment resulted in minimal changes in ethylmorphine N-demethylase or benzo(a)-pyrene hydroxylase activities (Tables 10 and 11). In the Long-Evans strain, ethanol-pretreatment caused a small but significant decrease in the rates of metabolism of ethylmorphine and benzo(a)pyrene (Table 11). The rates of metabolism of these two compounds were also minimally affected in the livers of the two strains of ethanol-treated mice (Tables 12 and 13). Thus the metabolism of these two substrates, which are generally used to monitor induction by phenobarbital and 3-methylcholanthrene, was not enhanced by ethanol treatment in all four strains of experimental animals studied.

Ethanol pretreatment consistently enhanced the O-deethylation of 7-ethoxycoumarin and the hydroxylation of aniline in livers of all four strains of rats and mice (Tables 10-13). There was a greater than 3-fold enhancement of aniline hydroxylase activity in livers of chronic ethanol-treated rats (Tables 10 and 11). In the two mouse strains, ethanol caused a 2-fold enhancement of aniline hydroxylase activity (Tables 12 and 13). 7-Ethoxycoumarin O-deethylase activity was increased 3-fold in the ethanol-treated rat strains (Tables 10 and 11), whereas 2- and

4-fold increases were observed in the C57 and A/J strains of mice, respectively (Tables 12 and 13).

In summary, the above data demonstrate that ethanol differs in inducing properties, when compared to the properties of the two classical hepatic microsomal enzyme inducers, phenobarbital and 3-methylcholanthrene. The shift in the absorbance maxima to longer wavelengths in the CO-difference spectrum observed in ethanol-treated animals is in contrast to the shift towards shorter wavelengths observed in rats pretreated with 3-MC, or no shift in PB-treated rats (99). Another contrast was the minimal effects observed in the metabolism of ethylmorphine and benzo(a)pyrene, whereas several fold increases were observed in hepatic 7-ethoxycoumarin O-deethylase and aniline hydroxylase activities in the ethanol-treated animals.

The electrophoretic mobilities, as determined by SDS-PAGE, of the various microsomal preparations utilized to study the spectral and catalytic properties of cytochrome P-450, were determined and the results are presented in Figs. 12 and 13. Wells B and E in Fig. 12 show protein-staining bands of solubilized microsomes from control Sprague-Dawley and Long-Evans rats. Several major bands were detected in the 50,000-60,000 molecular weight region where cytochromes P-450 generally migrate. Wells C and D show the protein-staining patterns of solubilized microsomes from ethanol-treated Sprague-Dawley and Long-Evans rats, respectively. Ethanol pretreatment appeared to produce a marked increase in the intensity of the protein bands in this molecular weight region. Mice microsomal suspensions were also subjected to SDS-PAGE (Fig. 13). Wells B and E in Fig. 13 show protein-staining bands of microsomes from C57 and A/J mice fed the control liquid diet. The protein-staining in well E is

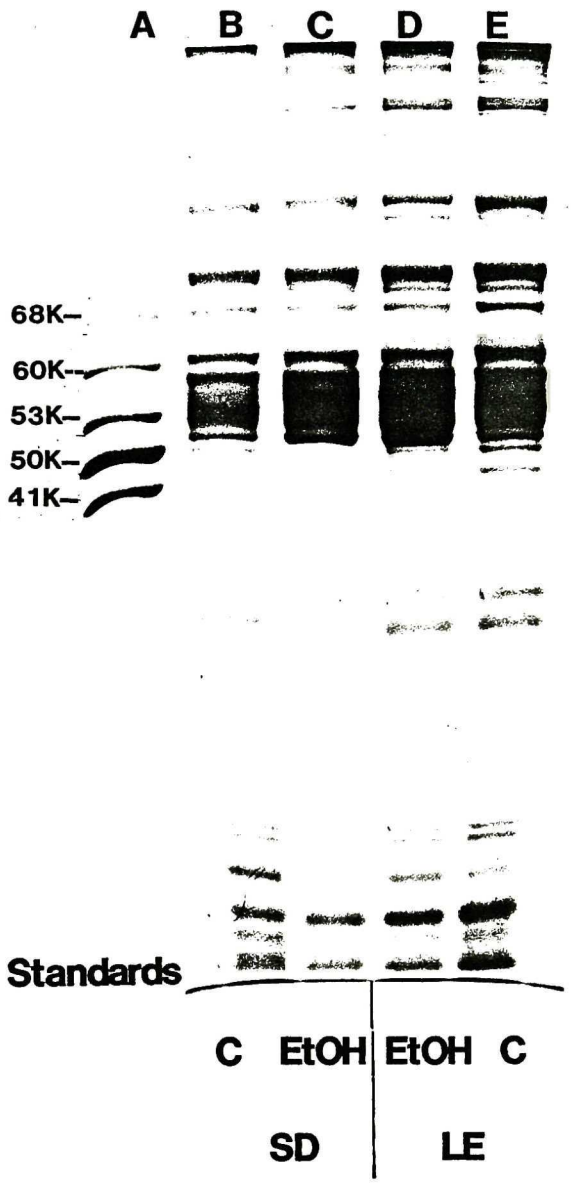


Fig. 12. SDS-PAGE of liver microsomes from chronic ethanol (EtOH)-treated Sprague-Dawley (SD) and Long-Evans (LE) rats. The animals were fed control or ethanol-containing liquid diets as described in the Methods section. Aliquots of microsomal suspensions, containing 100 µg of protein, were treated and subjected to slab gel electrophoresis as described in the Methods section. Wells B and C contained liver microsomes from control and ethanol-treated Sprague-Dawley rats, respectively; wells D and E contained liver microsomes from ethanol-treated and control Long-Evans rats, respectively. Well A contained protein standards having known molecular weights: bovine serum albumin, 68,000; catalase, 60,000; glutamate dehydrogenase, 53,000; fumarase, 50,000; and alcohol dehydrogenase, 41,000.

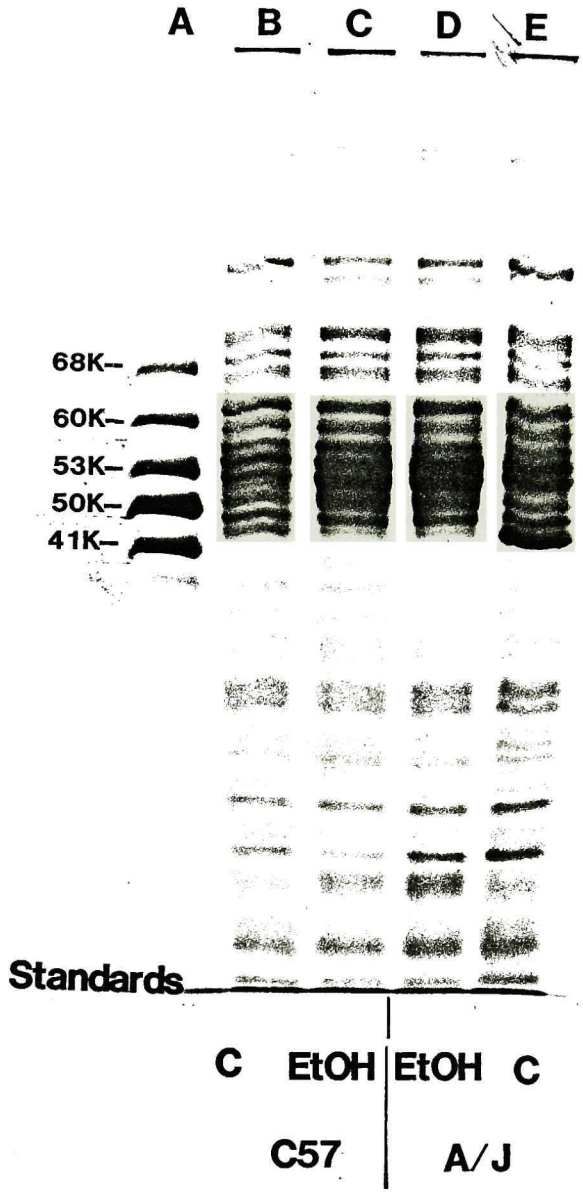


Fig. 13. SDS-PAGE of liver microsomes from chronic ethanol (EtOH)-treated C57 and A/J mice. The animals were fed control or ethanol-containing liquid diets, and liver microsomes were prepared for slab gel electrophoresis, as described in the Methods section. Microsomal suspensions, containing 100 µg of protein, were placed in each well. Wells B and C contained liver microsomes from control and ethanol-treated C57 mice, respectively; wells D and E contained liver microsomes from ethanol-treated and control A/J mice, respectively. Well A contained the protein standards described in legend to Fig. 12.

darker than in well B, possibly due to a slightly larger protein load applied to this well. Wells C and D show the protein-staining bands of microsomes from ethanol-treated C57 and A/J mice, respectively. The most striking feature of the protein-staining bands from the ethanol-treated mice was the pronounced staining of two bands at about 53,000 molecular weight.

In the present SDS-PAGE studies, the inducing effects of ethanol on the microsomal hemeproteins in rats and mice were further examined by staining the gels for heme-associated peroxidase activity. The densitometric results are shown in Figs. 14 and 15. In rats (Fig. 14) and in mice (Fig. 15), as shown in panels A and D, microsomes from animals fed the control liquid diet showed faint broad peroxidase activity bands slightly below the marker catalase standard of 60,000 molecular weight. In both rats and mice pretreated with ethanol, the hemeperoxidase activity, depicted in panels B and C of Figs. 14 and 15, showed marked increases in heme-staining in the same region. The above electrophoretic studies clearly demonstrate that in ethanol-treated rats and mice both protein and heme contents in the molecular weight region of cytochrome P-450 are increased.

3. The inductive properties of isopropanol in rats: In order to confirm the specificity of the inductive properties of ethanol reported above, experiments were carried out to determine the effects of another aliphatic alcohol, isopropanol, on the hepatic monooxygenase enzyme systems. Isopropanol was interesting because of its widespread use as an industrial solvent. Sprague-Dawley rats were administered isopropanol, 0.5 ml/kg, by gavage. In the acute studies, rats were killed 18 hr later. In the chronic studies, isopropanol, 0.5 ml/kg, was administered

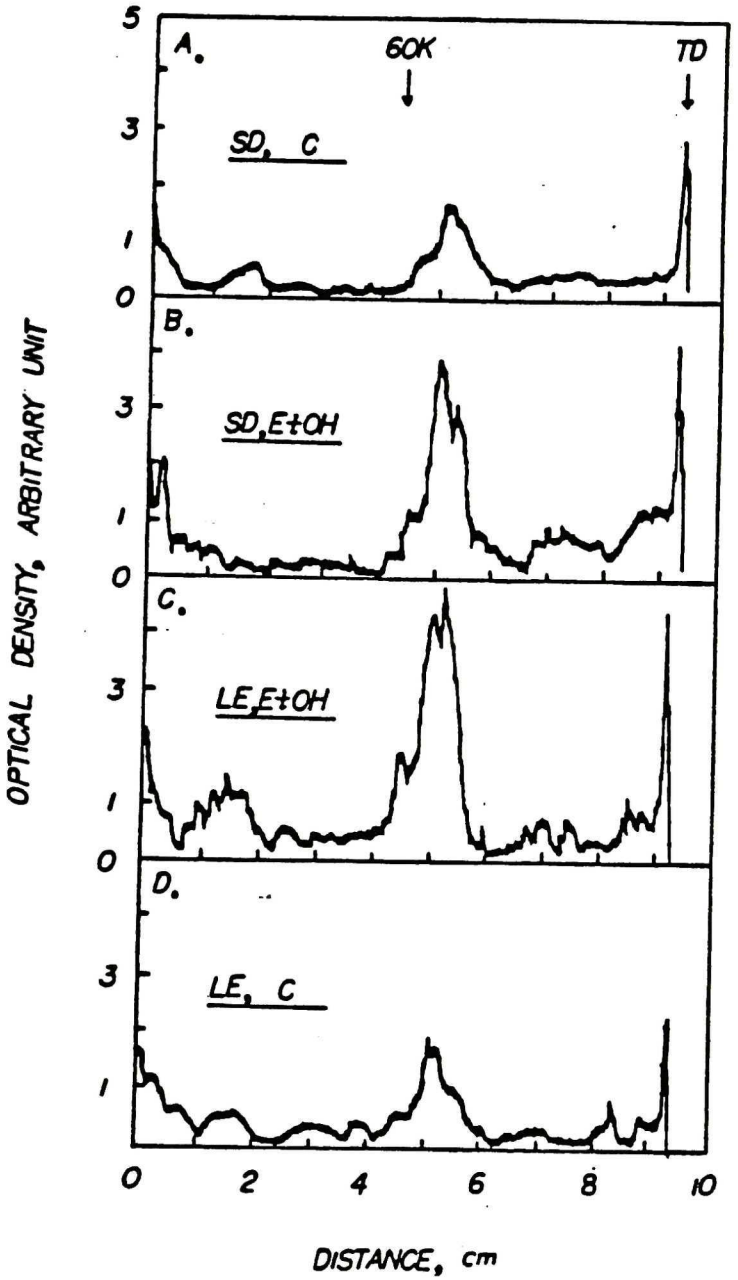


Fig. 14. Staining of peroxidase activity of liver microsomes from chronic ethanol (EtOH)-treated Sprague-Dawley (SD) and Long-Evans (LE) rats. The animals were fed the control or ethanol-containing liquid diets, and liver microsomes were subjected to slab gel electrophoresis, as described in the Methods section. The gel was stained for the heme-peroxidase activity by the method of Thomas et al. (76). A and B represent the densitometric tracings of peroxidase activities from control and ethanol-treated Sprague-Dawley rat liver microsomes, respectively; C and D represent the tracings from ethanol-treated and control Long-Evans rat liver microsomes, respectively. 60K represents the mobility of peroxidase activity peak of catalase, 60,000 molecular weight. TD represents the position of the tracking dye, bromphenol blue.

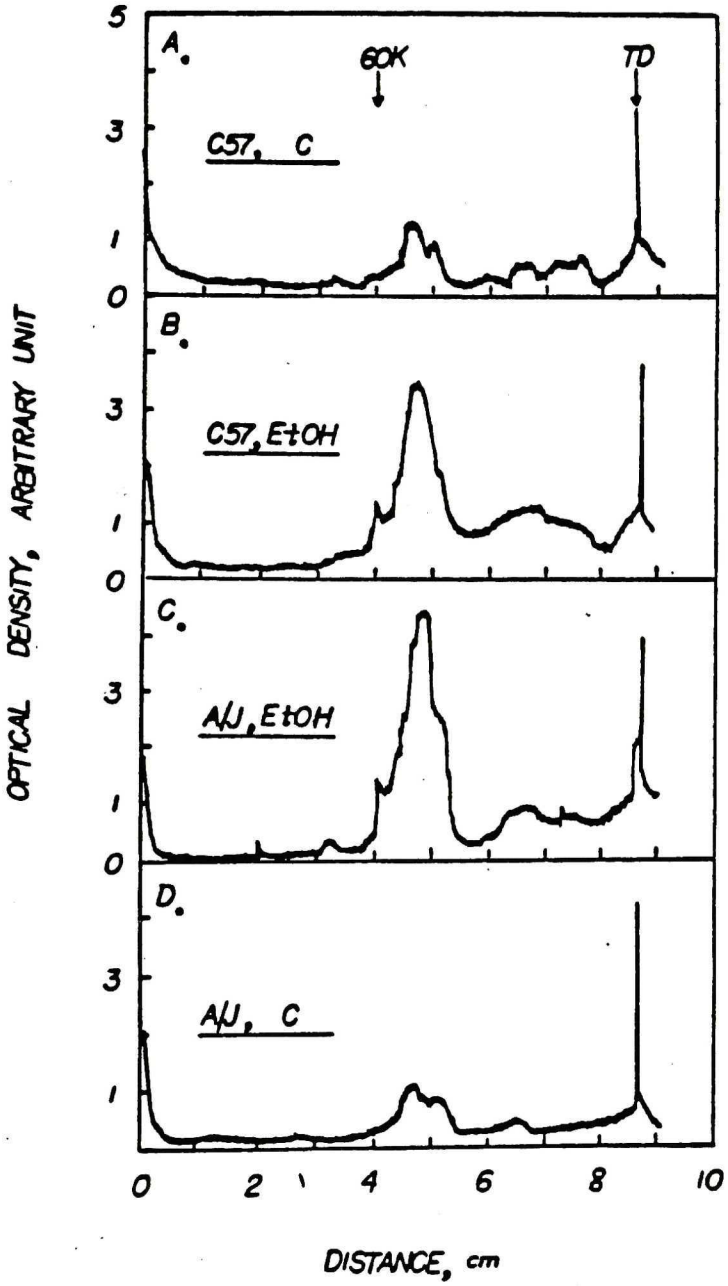


Fig. 15. Staining of peroxidase activity of liver microsomes from chronic ethanol (EtOH)-treated C57 and A/J mice. The animals were fed the control and ethanol-containing liquid diets, and liver microsomes were subjected to slab gel electrophoresis, as described in the Methods section. The gel was stained for the heme-peroxidase activity by the method of Thomas et al. (76). A and B represent the densitometric tracings of peroxidase activities from control and ethanol-treated C57 mice liver microsomes, respectively; C and D represent the tracings from ethanol-treated and control A/J mice liver microsomes, respectively. 60K represents the mobility of catalase, 60,000 molecular weight. TD represents the position of the tracking dye, bromphenol blue.

daily for 4 days and rats were killed 18 hr after the last dose of the alcohol. Hepatic microsomal components of the mixed-function oxidase system and cytochrome P-450-dependent enzymatic activities were assayed (Table 14). Pretreatment with isopropanol increased microsomal protein, even after a single dose of isopropanol. Acute or chronic treatment of the animals resulted in about a 20% increase in cytochrome P-450 content. The cytochrome P-450-dependent ethylmorphine N-demethylase and benzo[a]-pyrene hydroxylase activities were not altered by isopropanol pretreatment. In contrast, the isopropanol administration to rats caused a 3-fold increase in aniline hydroxylase activity and a 2-fold increase in 7-ethoxycoumarin O-deethylase activity (Table 14). The increases caused by isopropanol, in general, were slightly higher in the chronically treated animals; however, these differences were not statisically significant. These data demonstrate that the microsomal inductive properties of isopropanol, as with ethanol, were selective.

The electrophoretic mobilities, as determined by SDS-PAGE, of microsomal preparations utilized to study the effects of pretreatment of rats with isopropanol were determined and the results are presented in Fig. 16. Well B in Fig. 16 shows the protein-staining bands of solubilized microsomes from control rats. Several protein bands were detected in the 50,000 to 60,000 molecular weight region. Figure 16, well C shows that microsomes from livers of chronically pretreated animals showed no apparent increase in protein-staining in this molecular weight region. To further substantiate that the above changes in protein staining were associated with cytochrome P-450, the gels were stained for heme-associated peroxidase activity (Fig. 17). A protein standard catalase of 60,000 molecular weight was placed in well A. In control animals, more

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described in the Methods section. Well A contained protein standards having known molecular weights: bovine serum albumin, 68,000; catalase, 60,000; glutamate dehydrogenase, 53,000; fumarase, 50,000; and alcohol dehydrogenase, 41,000.

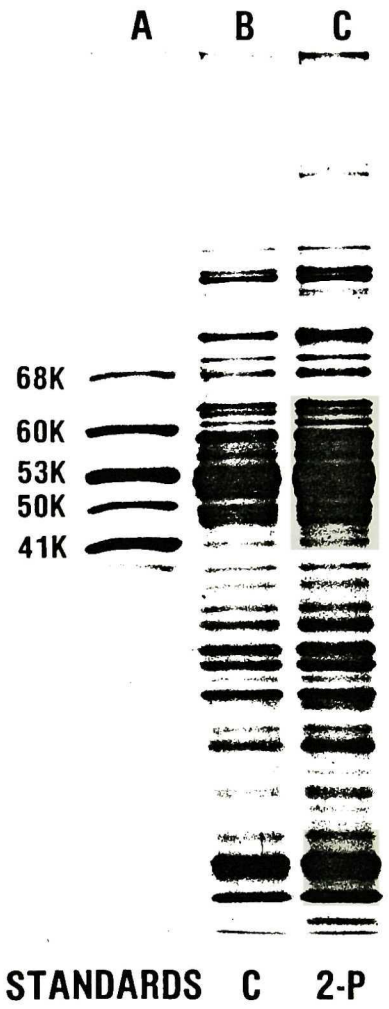

Fig. 16. SDS-PAGE of liver microsomes from isopropanol (2-P)-pretreated Sprague-Dawley rats. Rats were pretreated as described in the footnote in Table 14. Aliquots of microsomal suspensions, containing 50 μg of protein, were treated and subjected to slab gel electrophoresis as described in the Methods section. Well A contained protein standards having known molecular weights: bovine serum albumin, 68,000; catalase, 60,000; glutamate dehydrogenase, 53,000; fumarase, 50,000; and alcohol dehydrogenase, 41,000.

than one peroxidase activity band was observed in the region slightly below 60,000 molecular weight (Fig. 17, well B). Well C shows that chronic isopropanol pretreatment caused no apparent increase in heme-associated staining in the same region.

In comparison with the catalytic data reported above, no definitive conclusions can be drawn from the electrophoretic data obtained with isopropanol-treated rats. This was most likely, because isopropanol pretreatment elicited only an 18% increase in the specific content of cytochrome P-450 (Table 14).

4. The effects of ethanol on the microsomal epoxide hydrolase activity: Since ethanol and isopropanol selectively increased hepatic monooxygenase activities, it was of interest to determine the effect of ethanol on another oxidative enzyme which is not cytochrome P-450-dependent. Microsomal epoxide hydrolase activity was measured in livers from A/J and C57 mice and Sprague-Dawley rats (Fig. 18). Basal levels of epoxide hydrolase activity were similar for both strains of mice. The C57 were more highly induced by the chronic ethanol treatment than the A/J mice. Epoxide hydrolase activity was induced 33% in the A/J and over 2-fold in C57 mice and Sprague-Dawley rats by chronic ethanol treatment (Fig. 18). The rat control epoxide hydrolase activity was 2.5-fold higher than that found in the mouse strains.

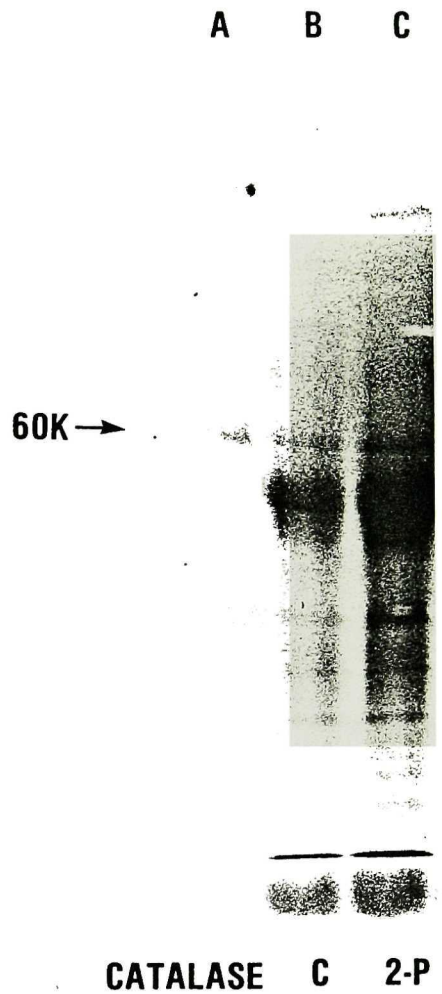

Fig. 17. Staining of peroxidase activity of liver microsomes from isopropanol (2-P)-pretreated Sprague-Dawley rats. Rats were pretreated as described in the footnote in Table 14. Aliquots of microsomal suspensions, containing 100 µg of protein, were treated and subjected to slab gel electrophoresis as described in the Methods section. The gel was stained for the heme-peroxidase activity by the method of Thomas et al. (76). Well A contained catalase, with a known molecular weight of 60,000.

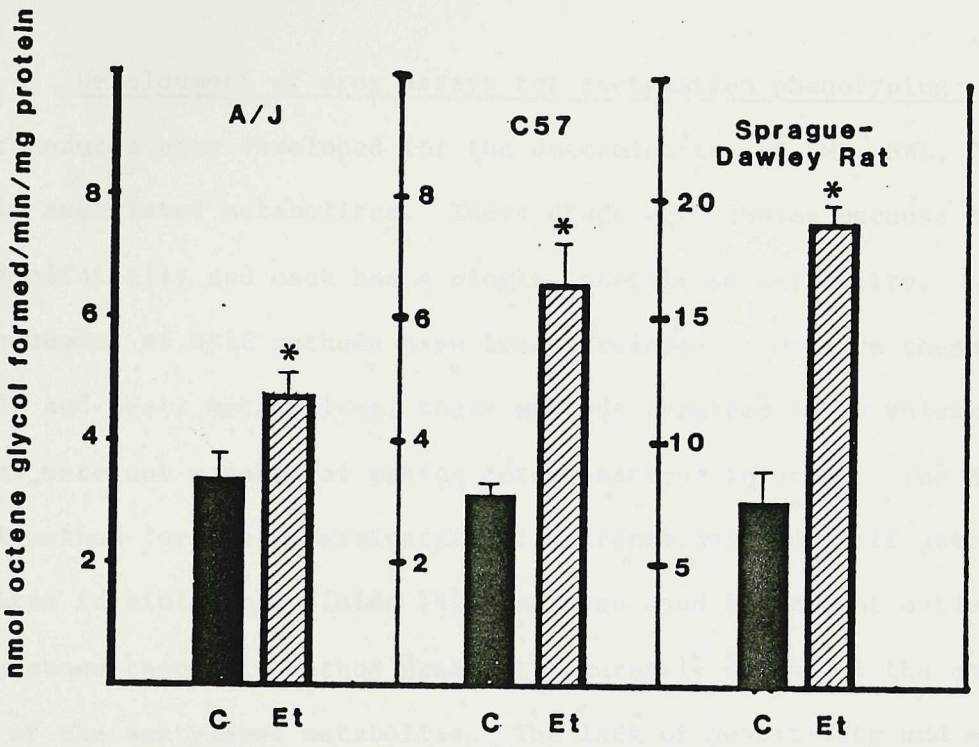

Fig. 18. Ethanol induction of epoxide hydrolase in A/J and C57 mice and Sprague-Dawley rats. Mice and rats were fed the ethanol-containing (Et) diet for 21 and 34 days respectively. Controls (C) received an isocaloric diet with maltose-dextrin substituted for ethanol derived calories. Each value represents mean ± S.E. for 5 animals. Asterisk represents value significantly different from respective control value ($p < 0.05$).

II. EFFECTS OF ETHANOL ON ACETYLATION CHARACTERISTICS IN A/J AND C57 MICE:

A. Development of drug assays for acetylation phenotyping: HPLC assay procedures were developed for the determination of SMZ, SNL, PA and their acetylated metabolites. These drugs were chosen because they are used clinically and each has a single, acetylated metabolite. Although a number of HPLC methods have been developed to measure these compounds and their metabolites, these methods required large volumes of blood and were not capable of making determinations in urine. The Bratton-Marshall method for the determination of sulfonamides and their acetylated metabolites in biological fluids (61) has been used but recent evidence (69) has shown that this method does not accurately determine the concentration of the acetylated metabolite. The lack of sensitivity and specificity of previously available methods led me to develop sensitive HPLC methods for assaying the compounds of interest.

Figures 19 and 20 show typical chromatograms obtained for SMZ and metabolite, and SNL and metabolite respectively, when mouse urine was chromatographed. The approximate retention times for internal standard SDZ (sulfadiazine), SMZ, and AcSMZ, under the conditions of their separation, were 2.8, 5.4, and 6.2 min., respectively. The approximate retention times for SDZ, SNL, and AcSNL, under the condition of this procedure were 3.7, 4.5, and 5.4 min., respectively. No interfering peaks were found in the areas of interest.

In order to determine the linearity and sensitivity of the assays, plots of the peak height of parent or metabolite to the peak height of the internal standard versus concentrations of aqueous standards (Fig. 21 and 22) were done. All samples were taken through the same procedure and

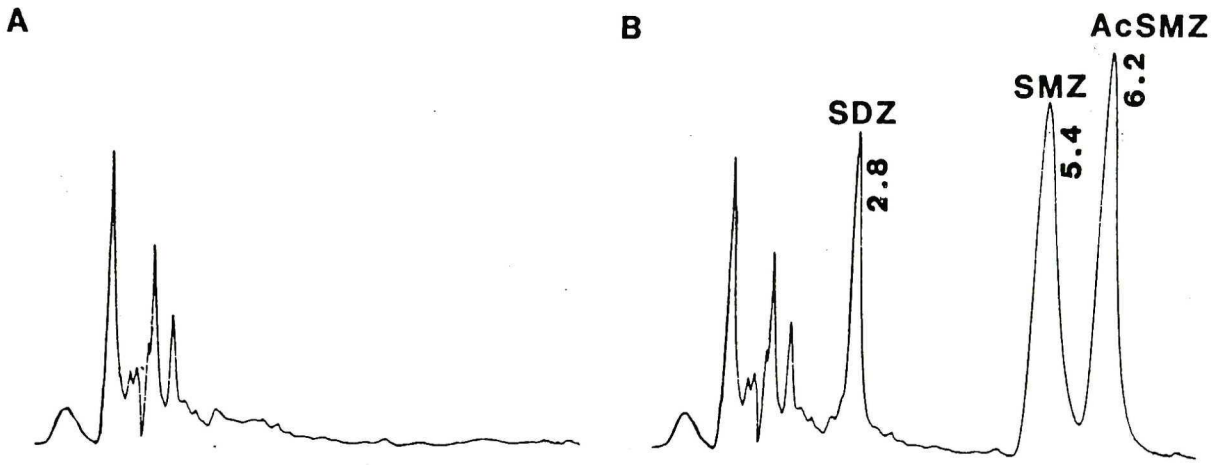

Fig. 19. Chromatogram of (A) blank urine and (B) urine containing 50 µg/ml each of sulfadiazine (SDZ), sulfamethazine (SMZ), and acetylsulfamethazine (AcSMZ). Column: C-18, 5 µm RAD-PAK; temperature: ambient; solvent: isocratic, 10 µmM sodium acetate buffer:methanol (68:32); flow rate, 2.0 ml/min; sample size: 20 µl; detector wavelength: 254 nm. Values on chromatogram represent retention times of the compounds of interest in min.

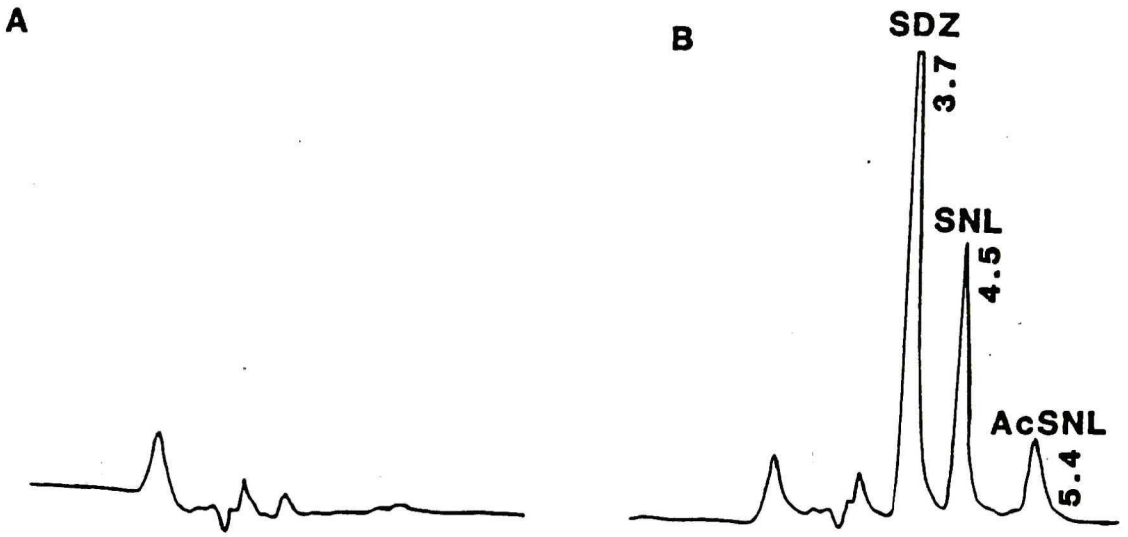

Fig. 20. Chromatogram of (A) blank urine and (B) urine containing 50 µg/ml each of sulfadiazine (SDZ), sulfanilamide (SNL), and acetylsulfanilamide (AcSNL). Chromatographic conditions were similar to those described in legend to Fig. 19 except flow rate was 1.0 ml/min and pressure was 1,000 psi. Values on chromatogram represent retention times of the compounds of interest in min.

resulted in linear curves to at least 100 µg/ml of the various compounds. The sulfonamides were reproducibly detectable and quantifiable in concentrations as low as 0.5 µg/ml in urine. Average analytical recoveries in urine (compared with the results of aqueous standards of equal concentration taken through the procedure) were 99.9% for each of the sulfonamides and metabolites over the range of concentrations examined.

When mice were administered SMZ, HPLC chromatographs of urine demonstrated the presence of a peak in addition to the peaks for SMZ and AcSMZ. This unknown peak eluted at 2.12 min, whereas SMZ and AcSMZ eluted at 5.4 and 6.2 min respectively. The column fraction corresponding to the unknown peak was collected and subjected to the Bratton Marshall procedure for the determination of sulfonamides. This fraction did not give a positive Bratton Marshall test indicating that the compound was not a free sulfonamide. However if this fraction was acid hydrolyzed, a positive reaction to the Bratton Marshall procedure was found indicating that the unknown peak might be a acetyl, glucuronide, or sulfate conjugate. In attempts to determine if the unknown compound was a conjugate, the urine containing the unknown was treated with β-glucuronidase (2.5 units/ml) or arylsulfatase (1 unit/ml), respectively, flushed with nitrogen for 5 min, and incubated in sealed tubes overnight at 37°. The samples were then centrifuged for 5 min at 3,000 x g and the supernatants removed for HPLC analysis. These enzymatic hydrolytic procedures did not change the peak height or retention time of the unknown peak indicating that the unknown peak was neither a glucuronide or sulfate conjugate. When the urine containing the unknown compound was acid hydrolyzed, the unknown peak disappeared from the HPLC chromatogram; however a new peak appeared which eluted at 1.03 min. In attempts to identify this compound several

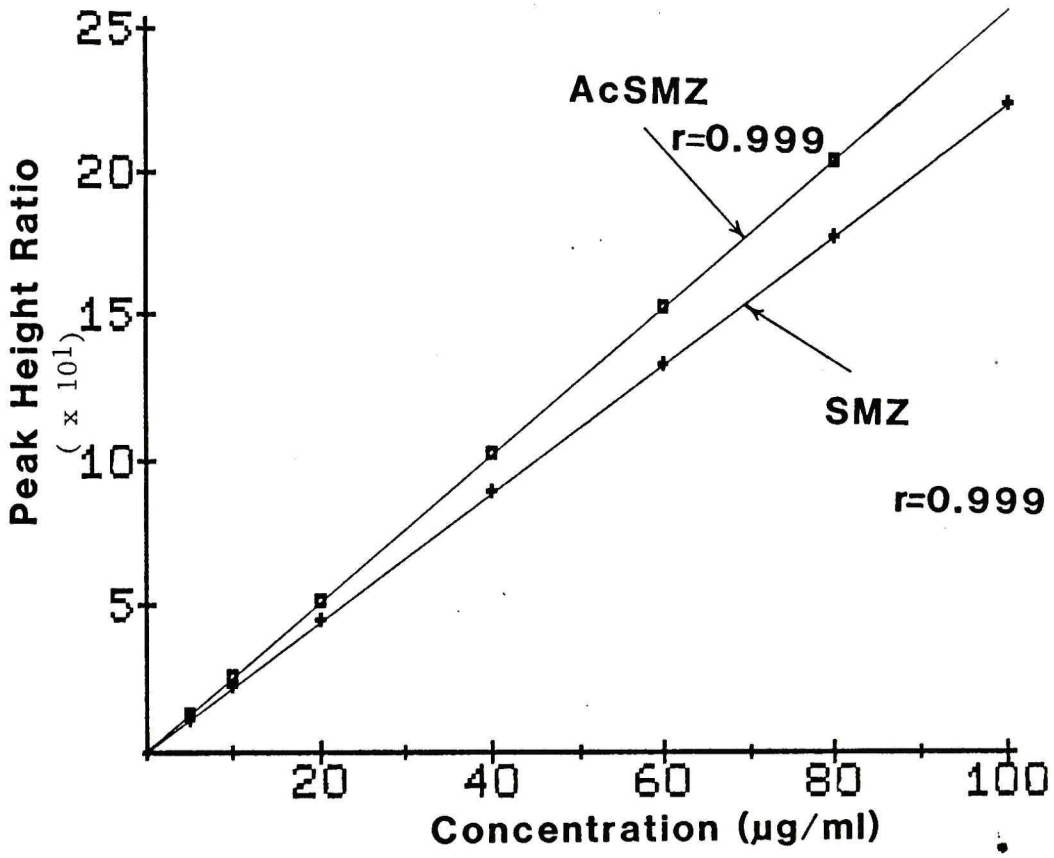

Fig. 21 Standard curves for sulfamethazine (SMZ) and acetylsulfamethazine (AcSMZ) in urine carried through the entire procedure. Curve plotted represents the peak height ratio (peak height of compound to peak height of internal standard, SDZ) versus the concentration of the standard compound. Each point represents the mean for 6 separate determinations. Peak height response was measured by a HP-3390 integrator/ plotter.

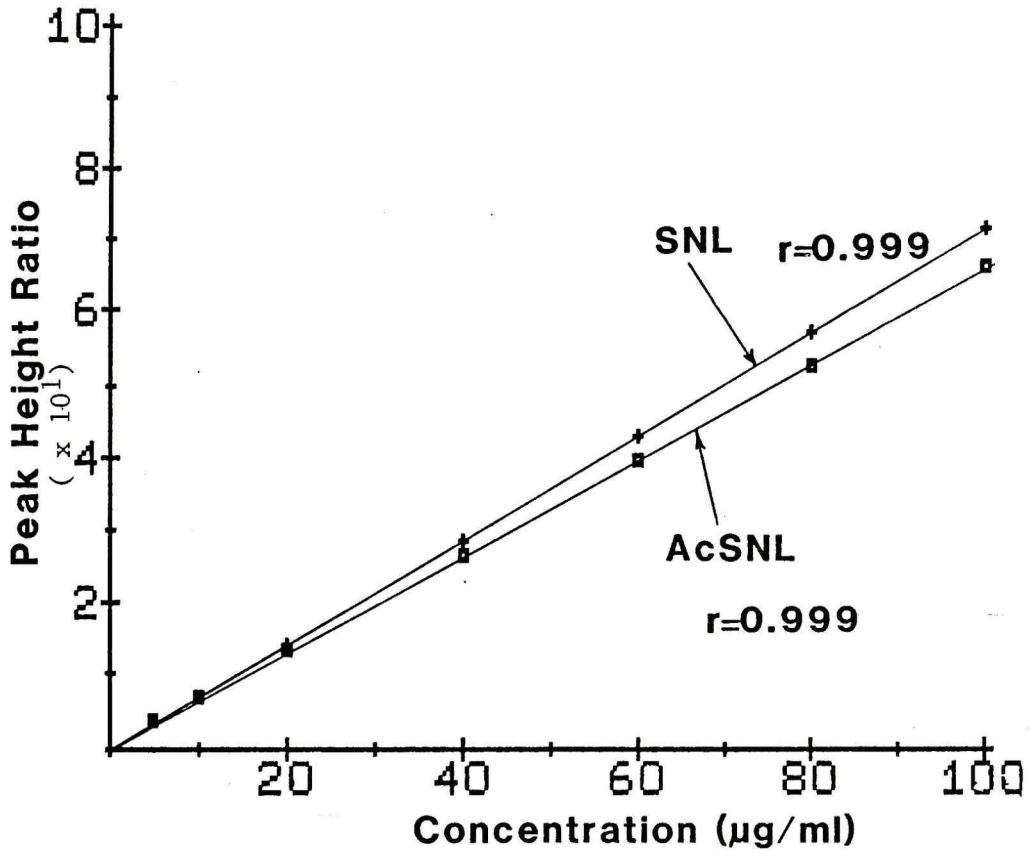

Fig. 22 Standard curves for sulfanilamide (SNL) and acetylsulfanilamide (AcSNL) in urine carried through the entire procedure. Curve plotted represents the peak height ratio (peak height of compound to peak height of internal standard, SDZ) versus the concentration of the standard compound. Each point represents the mean for 6 separate determinations. Peak height response was measured by a HP-3390 integrator/ plotter.

structually related compounds were purchased and run through the HPLC procedure. This list of compounds included most all of the sulfonamides commercially available and their potential metabolites such as sulfanilic acid and 2,4-dimethylaminopyrimidine. Only sulfanilic acid had a retention time identical to the acid hydrolyzed compound.

An HPLC method for the determination of PA and NAPA was also developed. Figure 23 illustrates a typical chromatogram for PA and NAPA in urine respectively. The approximate retention times for NAPA and PA, under the conditions of this procedure were 6.3, and 8.9 min., respectively. No interfering peaks were found in the areas of interest.

To determine linearity and sensitivity of the assay, the peak height ratio (determined against a fixed concentration of PA as an external standard in conjunction with a PA standard curve) versus concentration of the compounds were plotted (Fig. 24). All samples were taken through the same procedure and resulted in linear curves to at least 100 µg/ml for the various compounds. The compounds were reproducibly detected and quantified in concentrations as low as 0.5 µg/ml in urine. Average analytical recoveries from urine (compared with the results of aqueous standards of equal concentration taken through the procedure) were 95.0% for PA and 98.0% for NAPA over the range of concentrations examined.

Day to day chemical stability and precision of assays were evaluated by analyzing aliquots of pooled frozen urine containing parent drug and metabolite. Two sets of urine samples were prepared for each compound. One set was prepared in sterilized urine, the other set was prepared from normally collected urine. The latter was done to determine any possible effects of microbial degradation of parent drug and/or metabolite. Both sterilized and non-sterilized urine aliquots were

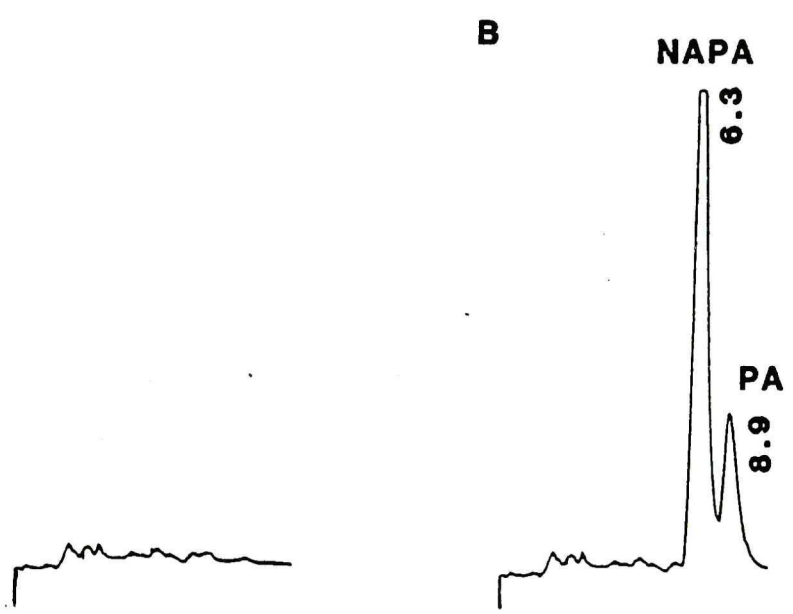

Fig. 23. Chromatogram of (A) blank urine and (B) urine containing 10 µg/ml each of procainamide (PA) and N-acetylprocainamide (NAPA). Column: C-18 5 µm RAD-PAK; temperature: ambient; solvent: isocratic, sodium acetate: acetic acid: water: acetonitrile: methanol 2.5:10/500/676/41 flow rate, 1.0 ml/min; pressure: 980 psi; sample size: 20 µl; detector wavelength: 254 nm. Values on chromatogram represent retention times of the compounds of interest in min.

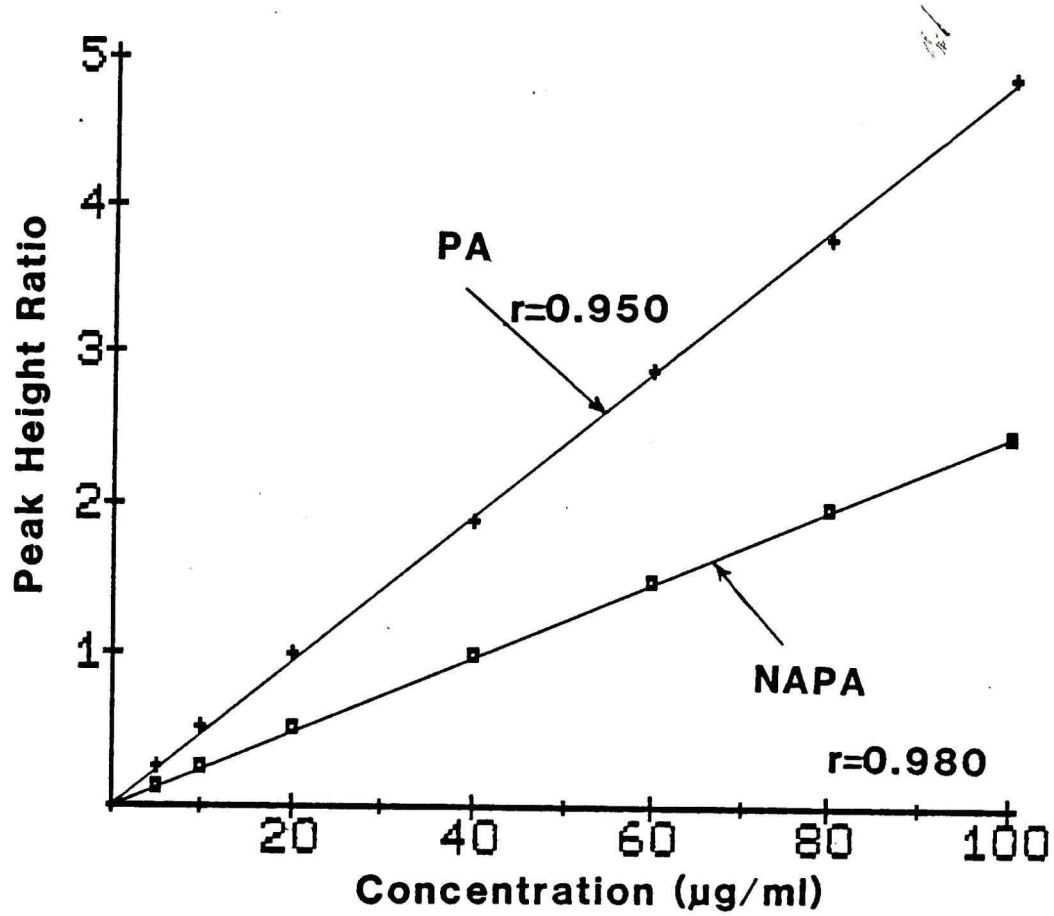

Fig. 24 Standard curves for procainamide (PA) and N-acetylprocainamide (NAPA) in urine carried through the entire procedure. Curve plotted represents the peak height ratio (peak height of compound to peak height of external standard, PA) versus the concentration of the standard compound. Each point represents the mean for 6 separate determinations. Peak height response was measured by a HP-3390 integrator/ plotter.

Table 15. Precision for HPLC analysis of sulfamethazine, sulfanilamide, procainamide and metabolites determined in untreated and sterilized urine standards over a 10 day period.

Compound Assayed	Precision (percent deviation)[a] Sterilized urine standards[b]	Non-sterilized urine standards[c]
Sulfamethazine/ acetylsulfamethazine	3.1 ± 0.4	2.8 ± 0.3
Sulfanilamide/ acetylsulfanilamide	3.4 ± 0.6	2.8 ± 0.6
Procainamide/ N-acetylprocainamide	2.6 ± 0.5	2.6 ± 0.9

[a] All urine standards were analyzed on the appropriate days in duplicate and the % deviation from the calculated standard was recorded. Precision is expressed as the mean ± S.E. of all the % deviations over the range of ratios prepared for a period of 10 days.

[b] Sterilized urine standards: Six A/J mice were placed in metabolic cages to collect urine for 24 hr. Mice were maintained on control liquid diet during the collection period. After 24 hr the cages were twice rinsed with 2.0 ml water and the total urine volume was brought up to 10 ml. All urine was pooled, placed in a boiling H_2O bath for 15 min to sterilize, cooled, and brought back up to the original volume and separated into 6 (10 ml) aliquots. To each aliquot parent drug and acetylated metabolite were added to make various ratios of parent to metabolite, such that the total amount of parent drug plus metabolite was equal to 2.5 mg. The SMZ and SNL standards were prepared with the following ratios of parent drug to metabolite: 0.40, 0.80, 1.20, and 1.60. The PA standards were prepared with the following ratios: 5.0, 10.0, 15.0, and 20.0. Standards containing only parent drug and only metabolite were also prepared for each compound group. Fresh standards were analyzed the first day, aliquots of the fresh standards were frozen at -10° C until analyzed on days 5, 8, and 10.

[c] Non-sterilized urine standards: Standards were prepared by adding the quantities of parent drug and metabolite used to prepare the each of the sterilized urine standards to each of six urine cups then collecting the urine in these cups for 24 hr as was done previously. Urine was brought up to 10 ml for each standard and an aliquot analyzed fresh and other aliquots analyzed on days 5, 8, and 10.

assayed for the compounds on days 1,5,8, and 10. The results of these studies are presented in Table 15. These studies showed that the compounds were stable in both urine prparations and were unaffected by storage or microbial interference. The experimental procedures were precise from day to day over the period examined.

SMZ and AcSMZ may crystallize in the kidney under certain conditions, such as periods when the animals are inadequately hydrated. I attemped to prevent this condition by feeding a liquid diet. To verify that crystallization did not occur, mice were administered SMZ (20-100 mg/kg, ip.) and renal tissue was examined at 24 hr for presence of SMZ and/or AcSMZ crystals. No evidence of the accumulation of crystals was apparent in renal tissue by histopathological examination.

B. Effects of acute ethanol administration on rates of acetylation of the various test drugs:

1. Effects of acute ethanol administration on apparent plasma half-life for the various test drugs: Sprague-Dawley and Long-Evans rats and A/J and C57 mice were administered ethanol, 3g/kg ip., acutely, followed with a challenge dose of SMZ (20 mg/kg, ip.) 30 min later. The apparent plasma half-life of SMZ was determined in each strain from the plasma elimination curves (Fig. 25). The apparent half-life of SMZ, 6.0 hr in Sprague-Dawley rats, was not significantly different from the apparent half-life of 6.7 hr obtained with Long-Evans rats. Acute ethanol treatment resulted in SMZ half half-lives of 4.0 and 4.5 hr in Sprague-Dawley and Long-Evans rats. The decrease in the apparent plasma half-life of SMZ elicited by ethanol was 33.3 and 32.8% in Sprague-Dawley and Long-Evans rats, respectively (Fig. 25). These decreases were statistically significant ($p < 0.05$).

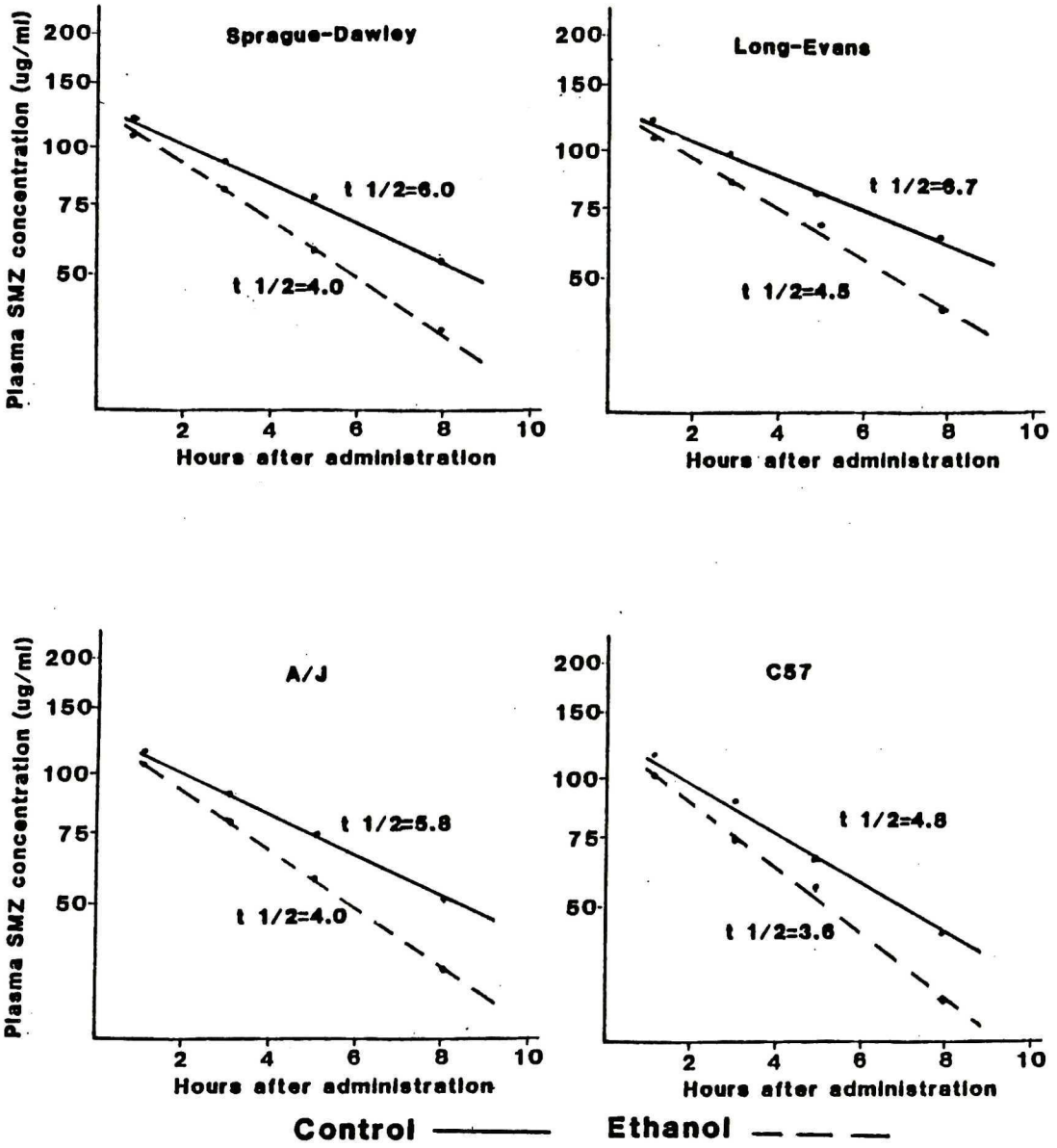

Fig. 25. Effects of acute ethanol pretreatment on plasma concentrations of sulfamethazine (SMZ) in Sprague-Dawley and Long-Evans rats, and A/J and C57 mice. Animals were treated with ethanol (3 g/kg, ip.) or an equal volume of water. Animals were then injected 30 min later with a challenge dose of the SMZ (20 mg/kg). Each value represents the mean for 5 animals.

The apparent plasma half-life of SMZ was significantly longer in the A/J mice than in the C57 mice with apparent plasma half-lives of 5.8 and 4.8 hr, respectively. This data is consistent with the classification of A/J mice as the slow acetylator strain and the C57 mice as the rapid acetylator strain. Both strains had significantly longer plasma half-lives for SMZ compared to the plasma half-lives of SNL, although no significant differences in the apparent half-life of SNL were noted between the two strains tested. The plasma half-lives of SNL were 2.1 and 2.0 hr in the A/J and C57 mice respectively. Acute ethanol treatment caused a significant 25-30% decrease in the apparent half-life of SMZ in both the A/J and C57 strain of mice. In contrast to data obtained with SMZ the acute ethanol administration did not significantly alter the apparent half-life of SNL in either strain of mice.

In summary, these data demonstrate that acute ethanol pretreatment increased the plasma elimination rate of SMZ as documented by the decreases in the apparent half-life of SMZ in both strains of rats and both strains of mice tested. It should also be noted that ethanol pretreatment effectively altered the apparent half-life of SMZ in the A/J mice such that this strain would be considered "rapid acetylators". The mean half-life of SMZ following acute administration of ethanol was similar to the mean half-life of SMZ obtained with the untreated, "rapid acetylator" C57 strain of mice (Fig. 25). On the other hand, SNL, a drug which is normally considered to be metabolized monomorphically, showed no effective change in the apparent half-life with ethanol pretreatment.

NAT activity was initially determined using the 9,000 x g supernatant prepared from livers of untreated Sprague-Dawley rats. The enzyme

fraction was incubated in the presence of SNL (90 μM) and AcCoA (0.8 mM) using various concentrations of protein. Enzyme activity remained in the range of 80-100 nM AcSNL formed/min over the concentration of 0.25-3.0 mg protein/ml. Rat liver NAT exhibited typical Michaelis-Menten kinetics as illustrated in Fig. 26 and by the Lineweaver-Burk plot (Fig. 26, inset). The Lineweaver-Burk plot was linear over the range of 0.05-0.8 mM AcCoA. The K_m for this enzyme was determined to be 0.162 mM with with a V_{max} of 119 nM/mg/min. Although Olsen (149) presented similar data he did not calculate K_m or V_{max} of this enzyme, but his data would indicate a K_m of 0.15-0.20 mM and a V_{max} of 120-130 nM/mg/min.

The above assay procedure was used to determine erythrocyte NAT activity in A/J and C57 mice. Mice were pretreated with ethanol, 3 g/kg ip., and a blood sample was drawn 1 hour later for determination of SMZ and PABA erythrocyte NAT activities. The reaction mixtures (90 μl) contained 50 μg protein of the enzyme source, 20 μl of a 10 mM solution of AcCoA, and 20 μl of 0.2 mM substrate solution (SMZ or PABA). Erythrocyte NAT exhibited low substrate activity for SMZ in both strains of mice. The strains also showed no polymorphism for this substrate based on the similarity of the NAT activities. SMZ erythrocyte NAT activity was uneffected by ethanol pretreatment in either strain (Table 16). A/J and C57 mice had 10- and 40-fold higher NAT activities for PABA as a substrate than for SMZ as a substrate. The C57 mice had significantly higher PABA erythrocyte NAT activity when compared to the PABA erythrocyte NAT activity determined in the A/J mice. Ethanol pretreatment did not alter PABA NAT activity in either strain.

The data from untreated mice was consistent with the previous data (11) that SMZ NAT activity in erythrocytes is low and therefore,

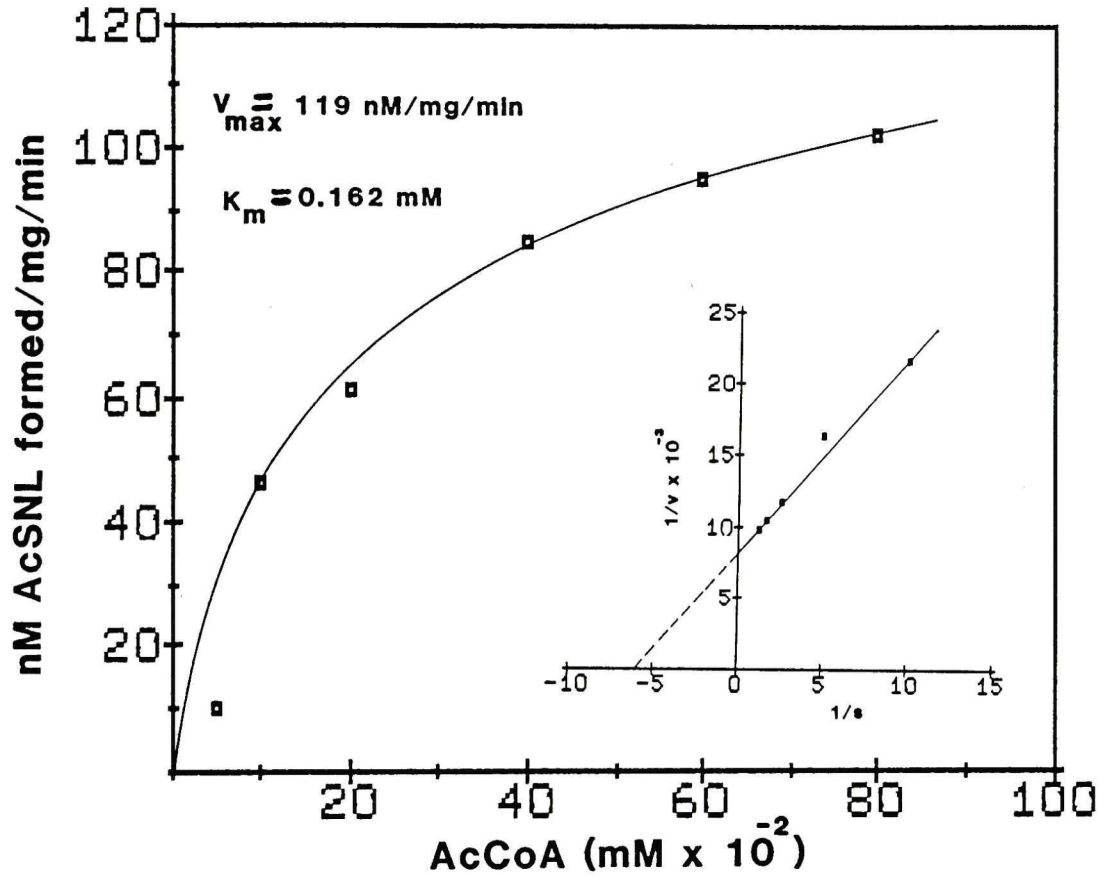

Fig. 26. Effect of AcCoA concentration on the rate of the N-acetyltransferase catalyzed reaction. Enzyme activity was determined in the 9,000 x g supernatant prepared from the livers of untreated Sprague-Dawley rats. Inset: Lineweaver-Burk plot.

Table 16. The effects of acute ethanol pretreatment on erythrocyte N-acetyltransferace activities in C57 & A/J mice[a]

Strain	Drug	Erythrocyte N-acetyltransferase activity (nmol acetylated product formed/mg protein/hr) Control	Ethanol
A/J	SMZ	0.020 ± 0.002	0.021 ± 0.004
	PABA	0.200 ± 0.020	0.210 ± 0.030
C57	SMZ	0.021 ± 0.004	0.020 ± 0.003
	PABA	0.800[b] ± 0.020	0.790[b] ± 0.030

[a] Animals were pretreated with ethanol, 3 g/kg i.p., and blood was drawn 1 hr later for determination of NAT activity. Each value represents the mean ± S.E. for 5 animals.

[b] Value significantly different from respective value determined in A/J strain ($p<0.05$).

possibly no polymorphism was observable in this species. Also substantiated by these studies was the polymorphism for PABA NAT activity in erythrocytes noted by Tannen and Weber (63). The studies presented indicate that acute ethanol does not alter the basal activities of the erythrocyte NAT using SMZ or PABA as substrates.

2. Effects of acute ethanol administration on the urinary excretion of SMZ, SNL, and PA: A/J and C57 mice were pretreated with ethanol, 3 g/kg ip., followed by a challenge dose of either SMZ, SNL, or PA (2.5 mg/mouse) 30 min later. The animals were then placed in individual metabolism cages for collection of urine. Urine collected over a 24 hr period was analyzed by HPLC and the amounts of parent compound and metabolite were quantified (Tables 17-19).

SULFAMETHAZINE: As shown in Table 17 control A/J mice excrete more SMZ and less AcSMZ in urine than the control C57 mice. Consequently, the ratio of AcSMZ to SMZ, is significantly lower for the A/J mice (0.50) than for the C57 strain (0.92). The ratio reflects the changes in both the parent compound and metabolite. The difference in this ratio of AcSMZ to SMZ has previously been used to distinguish rapid acetylator mice from slow acetylator mice (13). C57 mice were characterized as rapid acetylators because C57 mice generally had a urinary excretion ratio greater than 1.0 and the A/J mice generally had a urinary excretion ratio less than 1.0. In my studies C57 mice had a urinary excretion ratio close to 1.0 and the A/J mice had a ratio of 0.50 (Table 17). The differences are most likely due to the use of the Bratton-Marshal procedure which was unable to distinquish AcSMZ from the unknown peak which was detected in the urine by HPLC analysis. Therefore the acetyla tion ratios noted by Tannen and Weber (13) were higher than those determined

Table 17. Effects of acute ethanol pretreatment on urinary excretion of SMZ and AcSMZ in C57 and A/J mice[a]

Strain	Compound	Control (µg excreted/24 hr)	Ethanol
A/J	AcSMZ	248 ± 4	265[b] ± 7
	SMZ	499 ± 6	480[b] ± 5
	(AcSMZ/SMZ)	0.50 ± 0.01	0.55[b] ± 0.02
C57	AcSMZ	356[c] ± 8	368 ± 17
	SMZ	388[c] ± 8	379 ± 17
	(AcSMZ/SMZ)	0.92[c] ± 0.04	0.97 ± 0.09

[a] Mice were pretreated with ethanol, 3 g/kg ip., followed by SMZ injection (2.5 mg/mouse ip) 30 min later. Urine was collected for 24 hr and the amounts of parent compound and metabolite were quantified by HPLC. Each value represents the mean ± S.E. for 5 animals.

[b] Value significantly different from respective control value ($p < 0.05$).

[c] Value significantly different from respective value determined in A/J strain ($p < 0.05$).

by HPLC analysis.

A/J mice pretreated with a single dose of ethanol followed by SMZ injection excreted significantly higher amounts of AcSMZ and lower amounts of SMZ in the urine (Table 17). These changes were reflected in the change in the urinary excretion ratio of these compounds. The 10% increase in the urinary excretion ratio was moderate when compared to a 31% decrease in the apparent plasma half-life of SMZ in this strain after ethanol pretreatment (Fig. 25). The urine sample analyzed was a 24 hr urine sample. It is possible that the urinary excretion rate might have been greater in the initial period following the SMZ injection. In the fast acetylator C57 strain of mice, ethanol did not significantly alter the urinary concentation of AcSMZ or SMZ, nor did it change the urinary excretion ratio.

The peak height and retention time of the unknown peak was not significantly altered by ethanol pretreatment in either strain of mice ($p < 0.05$).

SULFANILAMIDE: Control A/J mice excrete significantly less AcSNL and more SNL in urine than do C57 mice (Table 18). This finding is also reflected in the larger urinary excretion ratio for C57 mice. Acute ethanol pretreatment did not change the amounts of SNL or AcSNL excreted in the urine or the urinary excretion ratio for the A/J mice. On the other hand, ethanol pretreatment caused small but significant changes in the concentrations of SNL and AcSNL in the urine of C57 mice (Table 18). The 18% change in the urinary excretion ratio is in contrast to the insignificant change in the apparent plasma half-life of SNL after ethanol pretreatment.

PROCAINAMIDE: Control C57 mice do not excrete different levels of

Table 18. Effects of acute ethanol pretreatment on urinary excretion of SNL and AcSNL in C57 and A/J mice[a]

Strain	Compound	Control	Ethanol
		(µg excreted/24 hr)	
A/J	AcSNL	1044 ± 14	1048 ± 21
	SNL	878 ± 14	873 ± 22
	(AcSNL/SNL	1.19 ± 0.03	1.20 ± 0.15
C57	AcSNL	1197[b] ± 11	1270[c] ± 25
	SNL	723[b] ± 12	650[c] ± 27
	(AcSNL/SNL)	1.66[b] ± 0.04	1.95[c] ± 0.11

[a] Mice were pretreated with ethanol, 3 g/kg ip., followed by SNL injection (2.5 mg/mouse ip) 30 min later. Urine was collected for 24 hr and the amounts of parent compound and metabolite were quantitated by HPLC. Each value represents the mean ± S.E. for 5 animals.

[b] Value significantly different from respective value determined in A/J strain ($p < 0.05$).

[c] Value significantly different from respective control value ($p < 0.05$).

NAPA and PA in urine compared to the A/J mice (Table 19). The differences are represented by the significantly different urinary excretion ratios. In contrast to the effects of acute ethanol pretreatment on SMZ excretion, acute ethanol pretreatment influences the excretion of NAPA in the C57 mice but not the A/J mice (Table 19).

C. Effects of chronic ethanol administration on rates of acetylation of various test drugs:

1. Effects of chronic ethanol administration on erythrocyte NAT activity: A/J and C57 were fed a liquid diet for 21 days as previously described in the Methods section. Controls received an isocaloric diet with maltose-dextrin substituted for ethanol derived calories. Blood samples were drawn for NAT determination 18 hr after last feeding for determination of erythrocyte NAT activities using SMZ and PABA as substrates. The results are presented in Table 20. Erythrocyte NAT activities from the chronic study were similar to those presented in the acute study. These studies suggest that erythrocyte NAT activity was unaffected in A/J and C57 mice by the acute or chronic administration of ethanol. Since NAT activity was unaffected in these studies, it may be concluded that ethanol does not function as a NAT enzyme inducer or a NAT enzyme inhibitor, but that the increases in acetylation activity found within the intact animal are more likely due to increases in the cofactor (AcCoA) level.

2. Effects of chronic ethanol pretreatment on urinary excretion of SMZ, SNL, and PA: For the day 0 determination the A/J and C57 mice were pretreated acutely with ethanol, 3 g/kg ip., followed by a challenge

Table 19. Effects of acute ethanol pretreatment on urinary excretion of PA and NAPA in C57 and A/J mice[a]

Strain	Compound	Control (µg excreted/24 hr)	Ethanol (µg excreted/24 hr)
A/J	NAPA	166 ± 18	168 ± 18
	PA	1370 ± 17	1367 ± 18
	(NAPA/PA)	0.12 ± 0.01	0.12 ± 0.01
C57	NAPA	136 ± 10	251[b] ± 19
	PA	1397 ± 11	1313 ± 41
	(NAPA/PA)	0.10 ± 0.01	0.19[b] ± 0.02

[a] Mice were pretreated with ethanol, 3 g/kg ip., followed by PA injection (2.5 mg/mouse ip) 30 min later. Urine was collected for 24 hr and the amounts of parent compound and metabolite were quantitated by HPLC. Each value represents the mean ± S.E. for 5 animals.

[b] Value significantly different from respective control value ($p < 0.05$).

Table 20. The effects of chronic ethanol pretreatment on erythrocyte N-acetyltransferace activities in C57 & A/J mice[a]

Strain	Drug	Erythrocyte N-acetyltransferase activity (nmol acetylated product formed/mg protein/hr) Control	Ethanol
A/J	SMZ	0.024 ± 0.003	0.015 ± 0.002
	PABA	0.274 ± 0.020	0.256 ± 0.026
C57	SMZ	0.028 ± 0.008	0.017 ± 0.002
	PABA	0.825[b] ± 0.020	0.813[b] ± 0.038

[a] Mice were fed liquid diet for 21 days. Controls received isocaloric diet with maltose-dextrin substituted for ethanol derived calories. Each value represents the mean ± S.E. for 5 animals.

[b] Value significantly different from respective value determined in A/J strain ($p<0.05$).

dose of either SMZ, SNL, or PA (2.5 mg/mouse) 30 min later. The animals were then placed in individual metabolic cages for collection of urine. Urine collected over a 24 hr period was analyzed by HPLC and the amounts of parent compound and metabolite were quantified. The mice were then placed on liquid control diet or liquid ethanol diet for the subsequent 21 days. On days 11 and 21 the same animals were placed in individual metabolic cages and allowed to feed on the liquid diets for 0.5 to 1 hr prior to the drug challenges. Animals were allowed to continue feeding on the liquid diets during the 24 hr urine collection period. Urine collected was analyzed as in the acute studies. The amounts of parent drug and metabolite were determined, as well as the urinary excretion ratio for the slow acetylator A/J mice and the fast acetylator C57 mice.

SULFAMETHAZINE: A/J mice chronically fed the ethanol-containing liquid diet excreted urine containing higher amounts of AcSMZ, lower amounts of SMZ, and had higher urinary excretion ratios on all 3 days assayed (Table 21). The results from day 21 for the chronically treated A/J mice were significantly different in all respects to results obtained initially on day 0. On day 21 both the control and ethanol-treated A/J mice were excreting significantly higher amounts of AcSMZ than each respective group did on day 0. Although there was an increase in acetylation ability for both groups, on day 21 ethanol still was capable of increasing the amount of AcSMZ excreted. The increased acetylation capability found in both groups on day 21 could be due to the weaning onto the liquid diets and the gradual changes in the diet over the course of the study. In contrast to the results obtained with the A/J mice, the urinary excretion of SMZ and AcSMZ was unaffected by the ethanol treatment in the C57 mice when challenged with a dose of SMZ on days 0,

Table 21: Effects of chronic ethanol pretreatment on urinary excretion of SMZ and AcSMZ in A/J mice[a]

Day	Compound	Control (µg excreted/24 hr)	Ethanol
0	AcSMZ	262 ± 7	294[b] ± 7
	SMZ	488 ± 11	450[b] ± 7
	(AcSMZ/SMZ)	0.53 ± 0.03	0.66[b] ± 0.02
11	AcSMZ	221 ± 18	288[b] ± 10
	SMZ	496 ± 24	457[b] ± 10
	(AcSMZ/SMZ)	0.43 ± 0.05	0.63[b] ± 0.04
21	AcSMZ	376 ± 10	405[b] ± 5
	SMZ	369 ± 11	352 ± 13
	(AcSMZ/SMZ)	1.03 ± 0.05	1.15[b] ± 0.07

[a] Mice were fed liquid diet for 21 days. Controls received isocaloric diet with maltose-dextrin substituted for ethanol derived calories. Animals were injected with SMZ (2.5 mg), placed in metabolic cages, and urine collected for 24 hr and the amounts of parent compound and metabolite were quantated by HPLC. Day 0 procedure was a repeat of the acute experiment previously described. Each value represents the mean ± S.E. for 5 animals.

[b] Value significantly different from respective control value ($p<0.05$).

11, and 21 (Fig. 27).

SULFANILAMIDE: Urinary excretion of SNL and AcSNL appeared to be unaffected by ethanol administration with the exception of Day 11 in the A/J mice (Table 22). In the acute studies, urinary excretion of SNL and AcSNL were also unaffected by the acute ethanol pretreatment (Table 20). In the chronically treated C57 strain, the mice had a higher urinary excretion ratio of AcSNL to SNL on days 0 and 11, but had a lower urinary ratio on day 21 (Fig. 28).

Acute or chronic ethanol pretreatments appears to selectively increase the amounts of the acetylated metabolite for the two sulfonamides studied. The increase in the urinary excretion of the acetylated metabolite was dependent on the mouse strain. Both the acute and chronic chronic treatment regimens increased the urinary excretion of AcSMZ in the slow acetylator A/J strain, but not in the rapid acetylator C57 strain. In contrast, both ethanol treatment regimens increased the excretion of AcSNL in the C57 strain, but not the A/J strain.

PROCAINAMIDE: As was previously observed in the acute ethanol studies, chronic ethanol pretreatment did not alter the amounts of PA or NAPA excreted in the urine of A/J mice on the days assayed (Table 23). Variations in values from day to day could be related to the nutritional status of the animals on each of the days assayed as previously mentioned. C57 mice acutely treated with ethanol had significantly higher urinary excretion ratios (Table 19), whereas the urinary excretion ratios were unaffected in the chronically pretreated animals (Fig. 29).

However, ethanol under these experimental conditions did not significantly alter the amounts of NAPA excreted in the urine. This could be due to the animals not consuming sufficient amounts ethanol

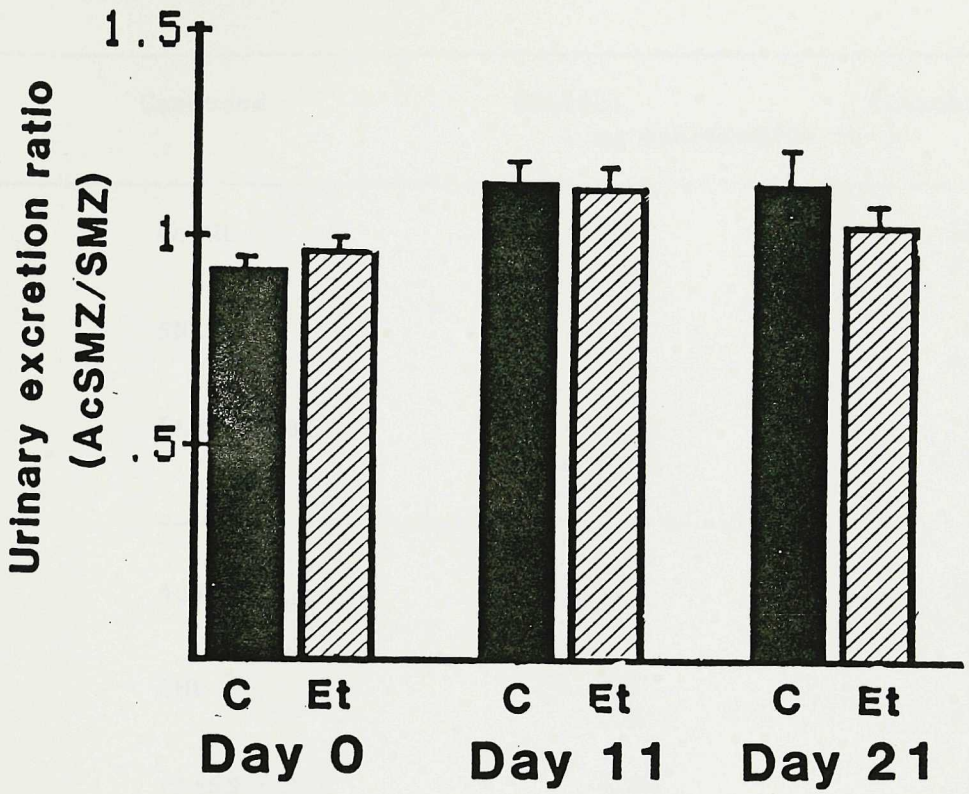

Fig. 27. Effect of chronic ethanol (Et) pretreatment on urinary excretion ratio of AcSMZ to SMZ in C57 mice. Control (C) animals received isocaloric diet with maltose-dextrin substituted for ethanol derived calories. Mice were challenged with SMZ as described in legend to Table 21. Each bar represents the mean ± S.E. for 5 animals.

Table 22: Effects of chronic ethanol pretreatment on urinary excretion of SNL and AcSNL in A/J mice[a]

Strain	Compound	Control (µg excreted/24 hr)	Ethanol (µg excreted/24 hr)
0	AcSNL	1087 ± 30	1040 ± 22
	SNL	833 ± 30	896 ± 21
	(AcSNL/SNL)	1.30 ± 0.08	1.16 ± 0.06
11	AcSNL	931 ± 11	1168[b] ± 14
	SNL	987 ± 8	751[b] ± 14
	(AcSNL/SNL)	0.94 ± 0.02	1.56[b] ± 0.05
21	AcSNL	1026 ± 16	988 ± 27
	SNL	894 ± 16	932 ± 27
	(AcSNL/SNL)	1.15 ± 0.04	1.07 ± 0.11

[a] Mice were fed liquid diet for 21 days. Controls received isocaloric diet with maltose-dextrin substituted for ethanol derived calories. Animals were injected with SNL (2.5 mg), placed in metabolic cages, and urine collected for 24 hr and the amounts of parent compound and metabolite were quantated by HPLC. Day 0 procedure was a repeat of the acute experiment previously described. Each value represents the mean ± S.E. for 5 animals.

[b] Value significantly different from respective control value ($p<0.05$).

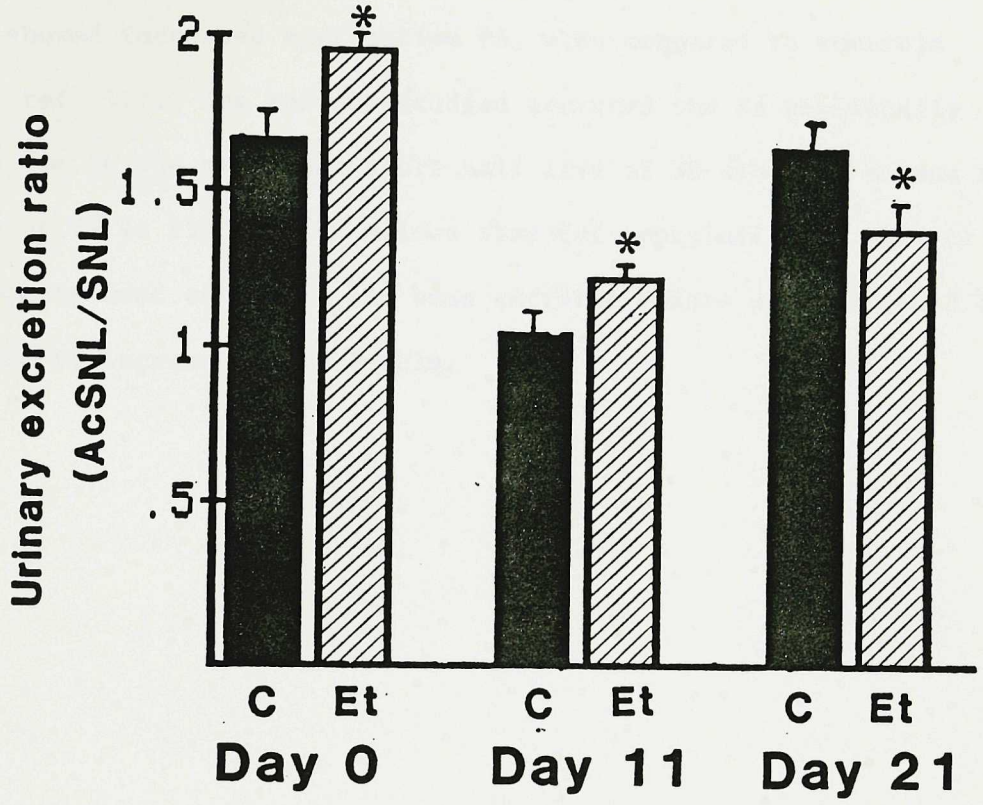

Fig. 28. Effect of chronic ethanol (Et) pretreatment on urinary excretion ratio of AcSNL to SNL in C57 mice. Control (C) animals received isocaloric diet with maltose-dextrin substituted for ethanol derived calories. Mice were challenged with SNL as described in legend to Table 22. Each bar represents the mean ± S.E. for 5 animals. Asterisk represents value significantly different from the respective control value ($p < 0.05$).

during the very short half life of PA, 30 min, to have any immediate effect on PA acetylation. These observations in mice treated with PA, appear to differ from the human situation. Humans administered PA and ethanol, showed increased acetylation PA, when compared to controls (Table 6, ref. 51). The rodents studied excreted the PA essentially unchanged due to the extremely short half live of 30 min. In humans the half live of PA is 155-180 min allows time for acetylation to NAPA to occur. In the rodents most of the PA had been excreted before ethanol could be metabolized to increase AcCoA levels.

Table 23: Effects of chronic ethanol pretreatment on urinary excretion of PA and NAPA in A/J mice[a]

Strain	Compound	Control (µg excreted/24 hr)	Ethanol
0	NAPA	166 ± 18	168 ± 18
	PA	1370 ± 17	1367 ± 18
	(NAPA/PA)	0.120 ± 0.011	0.123 ± 0.012
11	NAPA	86 ± 4	104 ± 10
	PA	1437 ± 12	1403 ± 29
	(NAPA/PA)	0.060 ± 0.007	0.074 ± 0.008
21	NAPA	140 ± 12	151 ± 15
	PA	1394 ± 12	1386 ± 30
	(NAPA/PA)	0.099 ± 0.009	0.109 ± 0.010

[a] Mice were fed liquid diet for 21 days. Controls received isocaloric diet with maltose-dextrin substituted for ethanol derived calories. Animals were injected with SMZ (2.5 mg), placed in metabolic cages, and urine collected for 24 hr and the amounts of parent compound and metabolite were quantated by HPLC. Day 0 procedure was a repeat of the acute experiment previously described. Each value represents the mea ± S.E. for 5 animals.

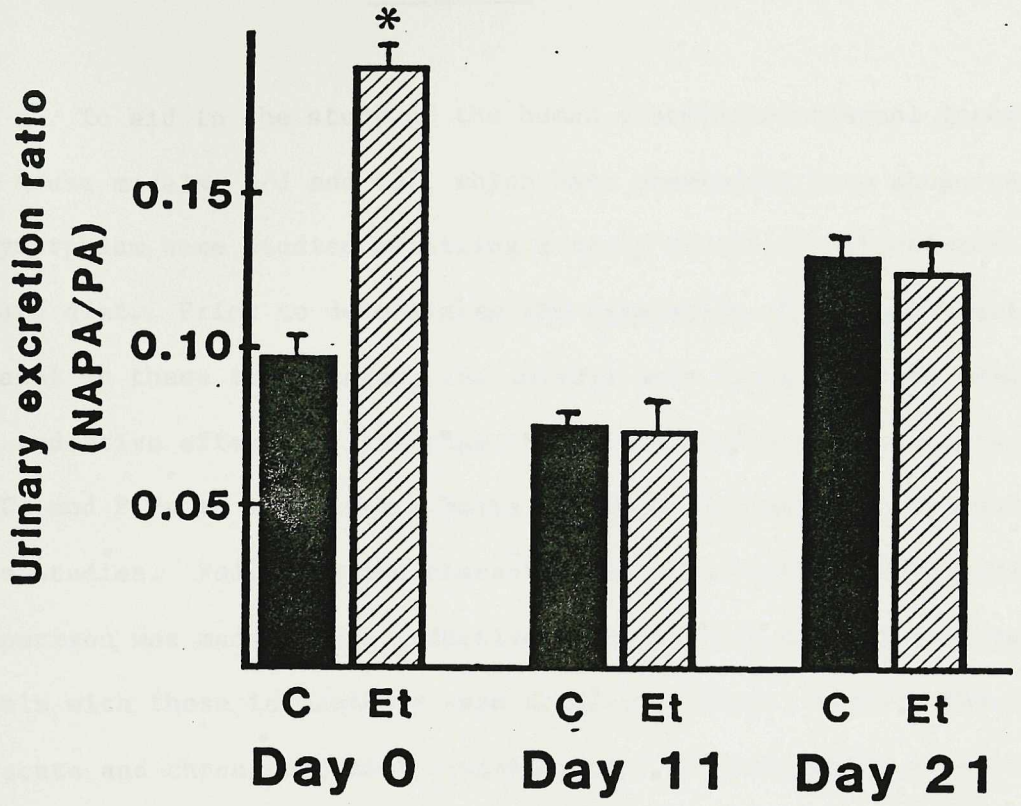

Fig. 29. Effect of chronic ethanol (Et) pretreatment on urinary excretion ratio of NAPA to PA in C57 mice. Control (C) animals received isocaloric diet with maltose-dextrin substituted for ethanol derived calories. Mice were challenged with PA as described in legend to Table 23. Each bar represents the mean ± S.E. for 5 animals. Asterisk represents value significantly different form respective control value ($p < 0.05$).

DISCUSSION

To aid in the study of the human acetylation-ethanol interaction, the mouse models, A/J and C57, which have previously been shown to exhibit polymorphism were studied utilizing a newly developed ethanol-containing liquid diet. Prior to determining the regulation of the acetylation by ethanol in these two strains, the animals were first characterized for the inductive effects of the classical microsomal enzyme inducers, PB, 3-MC, and PCBs to establish a basis of comparison with the ethanol induction studies. Following the characterization of hepatic monooxygenases, comparison was made of the inductive properties of ethanol in these mouse models with those in commonly used strains of rats. Finally the effects of acute and chronic ethanol administration on acetylation rates of several test drugs were determined in the mouse model. The results of each of these respective studies are discussed in the following sections.

A. The inductive properties of PB, 3-MC, and PCBs in mice and rats: The biotransformation of drugs and foreign chemicals is accomplished by a number of metabolizing enzymes which are characterized as either Phase I or Phase II enzymes. Phase I enzymes introduce polar groups into the parent compound thus providing a substrate for the Phase II conjugating enzymes to act upon. The oxidative biotransformations catalyzed by the liver microsomal enzymes are characterized as Phase I reactions, whereas the conjugation with an acetyl group as catalyzed by NAT is an example of a Phase II reaction.

Both monooxygenase (128) and acetylase (10, 11) activities have shown to be under genetic control in mice. The studies in this section were performed to characterize the inducing properties of the classical

inducing agents in the two strains of mice and to determine if polymorphism of microsomal enzyme inducibility also exists. Certainly, the poly morphism of benzo[a]pyrene hydroxylase inducibility among inbred strains of mice (130) could be just one of several possible microsomal enzymes subject to pharmacogenetic variation.

In recent years, several forms of cytochrome P-450 have been characterized. The microsomal monooxygenases differ in substrate specificities, spectral and immunological properties, and electrophoretic mobilities of these P-450s (104). The present studies substantiate previous reports (101,102,106) that in rat liver microsomes, the three best studied inducers markedly increased liver cytochrome P-450 content. PB and 3-MC caused preferential increases in the activity of ethylmorphine N-demethylase and benzo[a]pyrene hydroxylase activities, respectively. As expected, PCBs increased both monooxygenase activities.

In the A/J and C57 induction studies two polymorphisms of microsomal enzyme inducibility were found. The first polymorphism noted was for benzo[a]pyrene hydroxylase activity: the C57 mice were more "responsive" to the inducing properties of 3-MC than the A/J mice, consistent with previously reported data (130). The second polymorphism noted was for ethylmorphine N-demethylase activity; A/J mice were more "responsive" to the inducing properties of PB than the C57 mice, as refected in the inducibility of ethylmorphine metabolism. The PCBs mixture, Aroclor 1254, exhibited the inducing properties of both PB and 3-MC classes of inducers in the two strains of mice with no clear polymorphism noted for the enzyme activities determined. No obvious polymorphisms of basal monooxygenase activities were found, with both strains exhibiting similar activities.

Pretreatment with PB classically induced ethylmorphine N-demethylase as was found in the rats and in both strains of mice. 3-MC pretreatment produced typical P-448 type induction in the C57 mouse strain (128, 130). In contrast, the cytochrome P-450(s) induced by 3-MC in A/J mice showed different electrophoretic profiles and spectral properties from P-448 type induced in the C57 mice.

These data provide strong evidence for the genetic expression of the induction of cytochrome P-450-dependent monooxygenases in the two mouse strains, which were of interest for the subsequent acetylation studies. In rats the present studies confirmed previous catalytic, spectral, and electrophoretic data obtained with microsomes isolated from rats pretreated with the inducing substances PB, 3-MC, and PCBs. The results obtained strongly suggest differences in the regulatory mechanisms between species. The C57 and the A/J mouse strains appeared to show differences in inducibility of cytochromes P-450 and P-448, and the associated ethylmorphine N-demethylase and benzo[a]pyrene hydroxylase activities, respectively in liver microsomes.

These differences in inducibility may play an important role in the susceptibility of certain tissues and species to environmentally-derived toxicological agents. Agents which are metabolized by both the monooxygenase and acetylation pathways would be of the most concern. Certainly genetic variation in any cellular process involving biotransformation could also conceivably lead to atypical drug responses.

B. The inductive properties of alcohols in rats and mice: The ability of short chain aliphatic alcohols, such as ethanol, to induce hepatic microsomal mixed-function oxidases has been the focus of recent studies in some rodent species. In rats and rabbits, chronic feeding of

ethanol results in increases in hepatic cytochrome P-450 content and in aniline hydroxylase activity, with little or no change in ethylmorphine N-demethylase and benzo[a]pyrene hydroxylase activities (136, 155).

In the present studies, an attempt was made to determine the regulation of the cytochrome P-450-dependent monooxygenases by ethanol in the acetylator phenotypes of two inbred mouse strains. Based on the catalytic, spectral, and electrophoretic data it is apparent that the A/J (slow acetylator) and C57 (fast acetylator) strains of mice are similarly induced by chronic ethanol ingestion. Thus, the regulation of acetylator capacities in these mice would appear to be independent from the regulation of hepatic monooxygenases. This was expected since these two reactions are catalyzed by two different enzymes with different subcellular locations.

The uniformity of P-450-dependent enzyme induction by ethanol in the different species was rather unexpected. Other known enzyme inducers, such as PB and 3-MC, showed both species and, in the case of mice, strain differences. Previous studies have shown that in rats and rabbits, chronic feeding of ethanol results in increases in hepatic cytochrome P-450 content and in aniline hydroxylase activity (136, 155, 156). The present studies have demonstrated that chronic ethanol pretreatment resulted in very similar inducing effects in the two species, rats and mice as observed in the Sprague-Dawley and Long-Evans strains of rats and in the C57 and A/J strains of mice. In all 4 rodent strains chronic ethanol treatment consistently enhanced cytochrome P-450 content and aniline hydroxylase activity. In addition, the present studies demonstrated for the first time that 7-ethoxycoumarin O-deethylase activity was also enhanced by ethanol pretreatment.

The data presented in this report strongly suggest that ethanol induced a distinct form(s) of cytochrome P-450. Ethylmorphine and benzo(a)pyrene are poor substrates for this cytochrome P-450. In addition to differences in substrate specificities, the absorbance maximum in the CO-difference spectra of reduced liver microsomes from control animals, which occured at 450 nm, shifted to 451-452 nm when microsomes from ethanol-treated animals were studied. The electrophoretic data which showed marked increases in protein- and heme-staining bands in the 50,000-60,000 molecular weight region further support the conclusion that ethanol-pretreatment results in the induction of specific cytochrome(s) which are normally present in the untreated mice and rats, or the possible induction of a different species of the hemeprotein. In this regard, recent studies (156) have demonstrated the induction, in rabbits, by ethanol of a cytochrome P-450 isozyme having a molecular weight of 51,000 and a spectral absorbance maximum of 452 nm. This rabbit liver isozyme has an unusually high activity in the p-hydroxylation of aniline and in the oxidation of alcohols to aldehydes.

It would be of interest to determine if this form of cytochrome P-450 is also induced in livers of rats and mice chronically treated with ethanol. Further elucidation of the mechanism of induction of hepatic monooxygenases by ethanol requires the isolation and purification of this cytochrome and to determine its substrate specificities, and immunological reactivities of this specific form of hemeprotein in the two species of animals.

An extension of the above studies showed that another aliphatic alcohol which is widely used as an industrial solvent, isopropanol, possesses several of the inductive properties of ethanol. In animals

pretreated acutely or chronically with isopropanol, the total cytochrome P-450 content of liver microsomes was increased and the alcohol selectively induced the p-hydroxylation of aniline and O-deethylation of 7-ethoxycoumarin, while causing no significant changes in hepatic ethylmorphine N-demethylase and benzo[a]pyrene hydroxylase activities. These results differ from those of Sipes et al. (157) and Powis (158). In both studies, isopropanol treatment did not alter the amount of cytochrome P-450 measurable in hepatic microsomes. In the present studies acute and chronic treatment of rats with isopropanol increased total hepatic cytochrome P-450 by about 18%; this increase was statistically significant. However, under these experimental conditions, SDS-PAGE showed no apparent increase in specific protein- and heme-staining bands in the cytochrome P-450 region. These results indicate that the species of the hemeprotein induced by isopropanol and perhaps other aliphatic alcohols must normally be present in low concentrations, so that a several-fold increase in catalytic activity can be induced without markedly increasing total microsomal cytochrome P-450. The hemeprotein induced must therefore show a highly preferential selectivity towards aniline and 7-ethoxycoumarin.

These studies, therefore, appear to substantiate the postulate of Harris and Anders (159) that isopropanol (or ethanol) induces cytochrome P-450 and certain cytochrome P-450-dependent enzymatic activities. Sinclair et al. (150) have demonstrated that increases in cytochrome P-450 elicted by ethanol and certain other higher chain alcohols in cultured hepatocytes could be prevented by pretreatment of the cells with cycloheximide, an inhibitor of protein synthesis. In summary therefore, the inductive properties of isopropanol and ethanol differ markedly from those of the other microsomal drug-metabolizing enzyme inducers commonly

used experimentally, namely PB and 3-MC.

The therapeutic implications of ethanol and isopropanol-induced enzyme induction will be most obvious clinically for those drugs which compete for the hepatic drug metabolizing enzyme system. The inductive properties of these alcohols may also be important in environmental medicine, for workers who are occupationally exposed to those aliphatic alcohol solvents, and in the case of ethanol, to those who have a high chronic ethanol intake. Drugs with low therapeutic indicies would warrant drug monitoring to prevent possible side effects. An individuals' current drinking history, including past and present drinking habits should be taken into consideration due to the acute and chronic effects of ethanol on drug metabolism. The induction of the hepatic microsomal drug metabolizing system could increase the metabolism of xenobiotics, resulting in the formation of toxic metabolites. This may well be the effect seen in the observed interactions of ethanol with CCl_4 and acetaminophen, to produce hepatototoxicity (47,48). Additionally, the combined effects of cigarette smoking and ethanol consumption, two common social habits, presents a situation of two inducing agents acting in combination, possibly playing a role in the increased incidence of various forms of cancer and liver disease in individuals with these habits.

In the present alcohol studies, A/J and C57 mice and Sprague-Dawley rats treated chronically with ethanol had induced epoxide hydrolase activities. The Sprague-Dawley rats had the most highly induced epoxide hydrolase activity followed by the C57 and A/J mice, respectively. This induction of epoxide hydrolase may be of significance in carcinogenesis, since the epoxide metabolites formed by cytochrome P-450 mediated oxidations may be further metabolized to carcinigenic dihydrodiols intermediates

in the metabolism of polycyclic aromatic hydrocarbon carcinogens. Cigarette smoke contains a wide variety of polycyclic hydrocarbons whose metabolism to ultimate carcinogens is dependent on the activities of epoxide hydrolase and monooxygenases. Both these enzymic activities localized in the endoplastic reticulum of the liver and other non-hepatic tissues, such as lung, have been shown in the present studies to be induced by ethanol. The observed increase in incidence of cancer found among persons who smoke and consume significant amounts of ethanol could be partially due to this interaction.

C. Development of drug assays for acetylation phenotyping:

Phenotyping requires an analysis that insures specificity, speed, and accuracy. Most clinical phenotyping procedures meet these requirements. These clinical phenotyping procedures were not sufficiently sensitive for the purposes of the present studies. It should be noted that the commonly used methods for phenotyping human acetylation using the test drugs SMZ or dapsone, do not show a strictly bimodal distribution. There is a significant overlap in acetylation ability amoung individuals due to intraindividual variation from environmental influences. Because of the limitations of obtaining enough plasma and/or urine from mice and rats the development of highly sensitive assays was required.

1. The determination of sulfonamides: The HPLC procedures developed in the present studies for the separation of SMZ, SNL, and their respective metabolites were more sensitive then previously described methods (61,69). The new HPLC procedure required less than 1 µl of urine in contrast to 500 µl used in previous methods. The procedure was highly reproducible, stable, with short column retention times. The HPLC procedure required no derivatization, acid or heat hydrolysis, or

extraction of the samples, thereby simplifying sample preparation. The same system, with a small change in eluent rate (1 to 2 ml/min) enabled the measurement of both SMZ or SNL, and their respective acetylated metabolites.

In the present studies, the determination of SMZ and its metabolites in urine revealed not only SMZ and AcSMZ in urine but also a second metabolite which was diazotizable after acid and heat hydrolysis. This metabolite has not been previously reported in the two mouse strains when urine was analyzed by the Bratton-Marshall technique (13,63). It has been previously reported that the Bratton-Marshall technique does not distinguish N^4-amino metabolites from AcSMZ (153). This lack of specificity may have resulted in overestimation of the amounts of AcSMZ in mouse urine previously reported (13,68). Despite the lack of specificity for the metabolites of SMZ, the Bratton-Marshall technique is an accurate method for the determination of the parent sulfonamides. Therefore, the Bratton-Marshall technique was chosen to monitor plasma elimination rates and to determine the apparent half life of SMZ and SNL.

2. The determination of PA and NAPA: The HPLC procedure developed for the current studies for the determination of PA and NAPA was also more sensitive than previously described procedures (62,63). This procedure required only 1 µl of urine for the determination. The procedure provided accurate, reproducible, and stable measurements. The present studies utilized a procedure of comparing unknown peak heights against an external standard curve. The linearity of the standard curve was very reproducibly obtained. The HPLC injector system allowed precise volumes to be applied, a prerequisite for the use of external standard methods. Additionally, a Hewlett-Packard integrator/plotter utilizing an external

standard program was used to aid in calculations of the drug and metabolite concentrations.

Although in general an analytical method using an internal standard is preferable, an internal standard was not utilized in the present studies because suitable standards which chromatographed under these experimental HPLC conditions could not be found. Under the present conditions previously used internal standards coeluted with either PA or NAPA. In addition, other standards had prolonged retention times or were retained on the column.

The major advantage of HPLC procedures developed for the determination of the sulfonamides, PA and their associated metabolites was that very small samples of biological fluids were needed. These methods are preferable when studies using these test drugs are performed in small laboratory animals, due to the small sample sizes available. Additionally, these procedures would be applicable to forensic analysis here sample sizes are often quite limited.

D. Acetylation polymorphism and ethanol interaction: The primary goal of this section was to gain further insight into the human acetylation polymorphism and ethanol interaction. At the time that these studies were initiated, reports appeared in the clinical literature demonstrating that in humans, ingestion of ethanol enhanced the acetylation rate of SMZ (50) and PA (51). The C57 and A/J mice were chosen as the acetylation polymorphism models because these strains of mice were previously phenotyped as rapid and slow acetylators, respectively (10). Additionally, mice were shown to be adaptable to ethanol-containing liquid diets (52).

In the present studies the effects of acute and chronic ethanol pretreatments on acetylation polymophism in the C57 and A/J mice were

determined using SMZ, SNL, and PA as test drugs. Additionally, NAT activity was determined in hepatic and extrahepatic tissues.

1. Effects of ethanol administration on NAT activity in vitro: Using rabbit liver homogenates Weber and Cohen (19) studied the effects of increasing concentration of AcCoA on the rate of isoniazid acetylation and were able to show that NAT was activated by AcCoA and that the activated enzyme then reacted with the drug. Olsen (149) has reported similar findings with SMZ. In confirmation, the present studies found that increasing concentrations of AcCoA increased the V_{max} of the SNL acetylation reaction in rat liver homogenates. Using rat parenchymal cell suspensions, Olsen (149) recently reported that acetate or other precursors of AcCoA (pyruvate or citrate) increased the rate of SNL or PA acetylation. L(-)carnitine, which may enhance the translocation of AcCoA across the mitochondrial membrane, also increased the percent yield of the acetylated drug.

In species expressing variability in acetylating capacity, extrahepatic tissues contribute significant amounts of activity to the total acetylating capacity of the body, particularly in the genetically slow acetylator (64). Therefore, the effects of acute and chronic ethanol pretreatments on erythrocyte NAT activity would be of interest using SMZ and PABA as substrates since these substrates showed monomorphic and polymorphic acetylation, respectively, in the C57 and A/J mice. The present erythrocyte NAT studies showed that SMZ was a poor substrate for this enzyme as reflected in the low basal activity, and possibly because of this low activity no polymorphism was detectable with or without acute ethanol pretreatment. On the other hand, the erythrocyte preparation showed that PABA was polymorphically acetylated by NAT, and the C57 and

A/J mice were categorized as rapid and slow acetylators respectively, thus confirming an earlier report (11). Neither acute nor chronic ethanol pretreatment altered the acetylation of PABA by erythrocytes in the two mouse strains. Therefore, there is no apparent induction of NAT activity following chronic ethanol pretreatment of the mice.

No changes in acetylation rate was expected unless the enzyme had undergone some type of morphological or quantitative change. Under the condition of the assay, AcCoA was above saturating levels, and any elevated levels of AcCoA at the time of blood sampling would not increase the acetylation rate. A similar condition would exist if ethanol levels were elevated and the ethanol could be metabolized to AcCoA. Incidentally, the erythrocyte lacks the enzymes for ethanol metabolism. The only two other factors which could affect acetylation rate would be the drug concentration or a change in the amount or activity of the enzyme. Since the drug concentration was fixed and no change in acetylation activity occured with ethanol pretreatment the only conclusion which could be drawn is that is unlikely that ethanol produced any changes in enzyme amount or activity in either the C57 or A/J strains of mice.

2. Effect of acute ethanol administration on apparent half-life of test drugs: SMZ appeared to be eliminated from plasma at different rates in the two untreated mouse strains, namely the C57 and the A/J, fast and slow acetylators respectively. No such differences were noted between the control elimination rates in the two rat strains. The plasma elimination rate of PA was not determined due to the extremely short half-life of this drug. In animals treated acutely with ethanol, the plasma elimination rate of SMZ, but not SNL, was enhanced in both strains of mice tested. Also, similar findings were obtained using

Sprague-Dawley and Long-Evans rats.

The increased plasma elimination rates of SMZ following acute ethanol administration in the C57 (fast acetylators) and A/J (slow acetylators) mice mirrored the human situation for which increased plasma elimination rates of SMZ were observed when humans were administered ethanol acutely (50). The acetylation rate is regulated by the level of AcCoA in the vicinity of NAT (119,149). Ethanol metabolism has been shown to increase the cellular concentration of AcCoA (124,125). Therefore, the increased plasma elimination rate of SMZ may be due to the enhanced amount of AcCoA at the site of biotransformation resulting from ethanol metabolism.

3. Effects of acute ethanol administration on rates of acetylation of test drugs: The ratio of acetylated drug to non-acetylated drug in urine, as a single parameter, best represents the relative changes in the concentrations of the parent compounds and metabolites. The difference in the ratio of AcSMZ to SMZ has previously been used to distinguish the rapid acetylator C57 mice from the slow acetylator A/J mice (13). The present studies have confirmed such a genetic polymorphism in that a higher ratio of AcSMZ was found in the urine of C57 mice compared to the A/J mice (10). Previous studies have shown that SMZ acetylation polymorphism also exists in humans (8, 151).

The results obtained in the present studies have demonstrated for the first time acetylation polymorphism for SNL in the C57 and A/J mice. The urines of C57 mice show a higher ratio of AcSNL to SNL compared to the A/J mice, when a test dose of SNL was administered to either of mouse strains. This is in contrast to the situation in humans. Humans do not exhibit SNL acetylation polymorphism (8). In other studies,

although PA polymorphism is found in humans (51), under the present conditions PA acetylation polymorphism was not observed in the two mice strains, in confirmation of a previously reported study (68).

A single dose of ethanol to the slow acetylator A/J mice increased the urinary excretion ratio for SMZ. In contrast, acute ethanol pretreatment of the fast acetylator C57 mice did not alter the urinary excretion ratio of AcSMZ:SMZ. SNL urinary excretion ratio in the A/J mice was not altered by the acute ethanol pretreatment. On the other hand, the urinary excretion ratio for SNL in C57 mice was increased. Acute ethanol pretreatment increased the ratio of NAPA to PA in the C57 mice but not the A/J mice.

Acute ethanol pretreatment increased the urinary excretion ratio of acetylated to parent compound for SMZ in A/J mice and for SNL and PA in C57 mice. These results indicate that the enhanced acetylation of the various test drugs by ethanol show strain variability which was independent of phenotype or polymorphism. In comparison to mice, humans show more consistent increases in the amounts of acetylated metabolite in the urine after acute ethanol pretreatment for the test drugs SMZ (50) and PA (51). SNL has previously been shown to be metabolized monomorphically in humans (8), but the effects of acute ethanol pretreatment on the acetylation of this compound have not been investigated. On the basis of the hypothesis that in humans the acetylation rate is dependent on the concentration of AcCoA at the the site of NAT, and that ethanol may increase the cellular levels of AcCoA (via metabolism), it could be suggested that acute ethanol pretreatment in humans would effectively increase SNL acetylation, thus increasing the amounts of AcSNL in urine and decreasing the bioavailability of SNL.

4. Effects of chronic ethanol administration on rates of acetylation of various test drugs: In general, the effects of chronic ethanol pretreatment on acetylation characteristics were similar to those noted for acute ethanol pretreatment. Chronic ethanol pretreatment increased the amounts of the acetylated metabolite for the two sulfonamides studied. The increase in the urinary excretion of the acetylated metabolite was dependent on the mouse strain. Both ethanol treatment regimens increased the urinary excretion of AcSMZ in the slow acetylator A/J strain, but not in the C57 strain. In contrast, both ethanol treatment regimens increased the excretion of AcSNL in the C57 strain, but not the A/J strain. Chronic ethanol pretreatment did not alter the amounts of PA or NAPA excreted in the urine of A/J mice. C57 mice treated acutely with ethanol had significantly higher urinary excretion ratios for PA, whereas the urinary excretion ratios were uneffected in the chronically pretreated animals.

The present studies demonstrate that chronic ethanol pretreatment can increase the urinary excretion of acetylated metabolites. This increase is most likely due to the elevated levels of AcCoA from continuing ethanol consumption and subsequent metabolism. The increases found with the chronic ethanol pretreatment were strain and substrate dependent. No such chronic ethanol studies have been carried out in humans. It would be of clinical significance to determine the effects of chronic ethanol pretreatment on the acetylation of SMZ, SNL, or PA. Based on the present *in vivo* and *in vitro* studies, chronic ethanol consumption in humans may in certain instances increase the rates of acetylation of some drugs, and decrease the bioavailability of those drugs and increase the amounts of the acetylated compounds in the urine.

In the chronic ethanol studies, temporal variations in urinary excretion ratios from day to day were noted for all three compounds studied. These variations could be related to the nutritional status of the animals on each of the days assayed. These variations could be due to the weaning onto the synthetic diets and the gradual changes in the diet over the course of the study. Additionally, long term feeding of ethanol containing diets can have detrimental effects on liver function (86), and ultimately the acetylation capacity of the liver. This would be particulary true for the C57 mice which were shown to be less tolerant to rapid increases in ethanol content of the diet.

An additional factor affecting acetylation rate unique to PA as a test drug was the influence of its plasma half life. PA has a very short half life of 0.5 to 1.0 hr (63), and was most likely metabolized or eliminated before sufficient amounts of ethanol could be consumed and sufficently metabolized to acetate to affect acetylation. In short acetylation is a minor metabolic pathway for PA.

The effects of ethanol on plasma elimination rates were more pronounced than the effects of ethanol on the urinary excretion ratio of the compounds investigated. The variations noted may be due to a number of physiological parameters. The renal clearance of many compounds is pH dependent and metabolism may be affected by changes in the urine pH. The pK_a values of the compounds may be different and in a critical range with regard to normal values of urine pH, therefore the proportion of acetylated metabolite to parent compound would also be likely to be affected. Furthermore, urine flow rates also influence the renal clearance of drugs, and higher urine flow rates were noted during the feeding of the synthetic diet. Finally, the time over which urine is collected

could also influence the results due to different excretion rates amoung the compounds. A sample collected from 0-3 hr thus could give different value from one collected from 0-24 hr. In the present studies excretion rates were not determined, but the absolute amounts over the 24 hr period were recorded.

The present studies indicate that the A/J and C57 mice, under certain conditions, may model the increases in acetylation capacity seen during ethanol consumption in humans. However, the mouse model showed substrate and strain dependence which was independent of phenotype or previous polymorphism. The increases in acetylation noted in man or animals is most likey due to the metabolism of ethanol to AcCoA, the rate limiting cofactor for NAT. Acetylation polymorphism is important in drug therapy because of the serious drug toxicities associated with both the rapid and slow acetylator phenotypes. Any factor or condition which would affect acetylation is of clinical significance. In the present studies ethanol was shown to affect the rates of acetylation of certain drugs. Thus, in addition to affecting the Phase I oxidative enzymes, the present studies show that ethanol may alter a Phase II NAT catalyzed reaction.

Ethanol and isopropanol were shown to be consistent hepatic monooxygenase inducers, having inducing characteristics which were markedly different from the inducing agents PB, 3-MC, and PCBs. Additional novel findings reported here include the observation that epoxide hydrolase activity was induced by chronic ethanol pretreatment in rats and mice.

Due to the multiple effects of ethanol on drug metabolism, future studies involving the effects of ethanol on the metabolism of other arylamines, particularly the carcinogenic arylamines, would be most

interesting. The carcinogenic arlyamines, thought to be related to bladder cancer etiology (11), not only show acetylation polymorphism but may be metabolized by the Phase I hepatic monooxygenases as well as by epoxide hydrolase. All of these enzymes, known to metabolize the carcinogenic arylamines, were shown to be affected by ethanol in the present studies. Therefore, research on the etiology of bladder cancer could possibly benefit from additional research in this area. Various dietary and environmental conditions in addition to the genetic background of the individual phenotype may predispose an individual to certain such cancers. Certainly ethanol, as a dietary component, may be one such factor.

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